SATP (YaaH), a succinate–acetate transporter protein in Escherichia coli

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In the present paper we describe a new carboxylic acid transporter in Escherichia coli encoded by the gene yaaH. In contrast to what had been described for other YaaH family members, the E. coli transporter is highly specific for acetic acid (a monocarboxylate) and for succinic acid (a dicarboxylate), with affinity constants at pH 6.0 of 1.24 ± 0.13 mM for acetic acid and 1.18 ± 0.10 mM for succinic acid. In glucose-grown cells the ΔyaaH mutant is compromised for the uptake of both labelled acetic and succinic acids. YaaH, together with ActP, described previously as an acetate transporter, affect the use of acetic acid as sole carbon and energy source. Both genes have to be deleted simultaneously to abolish acetate transport. The uptake of acetate and succinate was restored when yaaH was expressed in trans in ΔyaaH ΔactP cells. We also demonstrate the critical role of YaaH amino acid residues Leu131 and Ala164 on the enhanced ability to transport lactate. Owing to its functional role in acetate and succinate uptake we propose its assignment as SatP: the Succinate–Acetate Transporter Protein.

Key words: acetate, bacteria, succinate, YaaH transport family, yeast.

INTRODUCTION

The acetate switch in Escherichia coli has been defined as a dynamic alteration of the cell physiology that occurs when acetate dissimilation equals its assimilation. This happens when cells are transitioning from a rapid growth phase, associated with the production and excretion of acetate, to a slower growth phase, supported by the import and utilization of acetate [1]. Acetic acid is a weak carboxylic acid that can dissociate in aqueous solution, and the amount of both anionic and lipophilic forms is dependent on the pH of the medium. As the pKₐ of acetic acid is 4.76, at neutral pH the acetate ion accounts for more than 99% of the acid form. Thus acetic acid-producing cells must possess plasma membrane transporters to efficiently export acetate in its anionic form out of the cell. Although the metabolic pathways associated with acetate dissimilation and assimilation in E. coli have been studied extensively (for a review see [1]), the identification of the plasma membrane transporters responsible for the export and/or import of acetate have not yet been fully elucidated. An acetate permease (actP), co-transcribed with acs (acyl-CoA synthetase) is involved in scavenging micromolar concentrations of acetate from the extracellular medium in E. coli [2]. The ActP transporter belongs to the Sodium:Solute Symporter Family (http://www.tcdb.org/) and is highly specific for short-chain aliphatic monocarboxylates, namely acetate, glycolate and propionate. However, Gimenez et al. [2] also claimed that, besides ActP, another transporter must exist in E. coli since the actP-deficient mutant strain displays low, but not null, rates of acetate transport. The identification of such a transporter remains to be done and our working hypothesis is based on the assumption that this function should be accomplished by the YaaH protein.

The YaaH family members are polytopic proteins with a predicted topology of six transmembrane segments, containing a conserved motif (N-P-[AV]-P-[LF]-G-L-X-[GSA]-F) located at the first putative transmembrane region of the N-terminus of the protein (http://www.tcdb.org/). Members of the YaaH family are found in archaea, eukaryotes and bacteria, with some members experimentally demonstrated as acetate transporters such as Ady2 (accumulation of dyad 2) in the yeast Saccharomyces cerevisiae [3] and AcpA (acetate permease) in the filamentous fungus Aspergillus nidulans [4]. Deletion of acpA in A. nidulans leads to reduced growth on acetate as a sole carbon source, at low concentration and in a high pH medium, which is fully restored upon reintroduction of the acpA gene. This gene is also required for the induction of the acetate assimilation pathways and direct measurement of acetate incorporation into germinating conidia confirmed that AcpA is fundamental for acetate uptake, especially at low substrate concentrations [4]. The acpA transcript level increases as the demand for acetate uptake increases and its expression is induced in the presence of several weak monocarboxylic acids such as glyoxylate, propionate, lactate, pyruvate and formate.

Acetic acid-grown cells of the yeast S. cerevisiae display activity for a monocarboxylate–proton symporter, shared by acetate, propionate and formate, dependent on the ΔPH across the plasma membrane [5], found to be associated with Ady2 expression. S. cerevisiae ADY2 is subjected to glucose repression and its expression occurs upon a shift from glucose medium to a non-fermentable carbon source, such as acetic acid [3]. The deletion of ADY2 results in the loss of mediated acetate uptake measured at pH 6.0, implying that this gene is essential for the acetate permease activity in S. cerevisiae [3]. The same protein was also hypothesized to be an ammonium exporter [6,7] since the null mutant displays a reduction in ammonia production when cells are growing in colonies [6], and the appearance of Ady2 at the plasma membrane correlated with ammonia release [7]. However, more recently, two independent teams have found that Ady2 is also implicated in lactic acid uptake [8,9]. It was...
demonstrated that the double mutant strain for Ady2 and for the lactate permease Jen1 (ady2Δ jen1Δ), but with the ADY2 gene expressed in a centromeric plasmid under the control of a strong constitutive promoter, displays Michaelis–Menten kinetics for the initial uptake rate of labelled lactic acid at pH 5.0 [8]. By using a laboratory evolution strategy of a S. cerevisiae jen1Δ strain, Kok et al. [9] found two genomic mutants in Ady2, L219V and A252G, with enhanced ability to grow on lactic acid. All this evidence supports a role for Ady2 as a monocarboxylate transporter located at the plasma membrane of yeast cells.

Although not directly shown to be functionally implicated in acetate uptake, other YaaH family members have been linked to acetate acid adaptation, such as Gpr1 (glyoxylate pathway regulator 1) in the yeast Yarrowia lipolytica and MA4008 in the archael species Methanosarcina acetivorans. In Y. lipolytica GPR1 mRNA expression is enhanced by acetic acid and although its deletion did not impair growth on acetic acid as a carbon source [10], mutations in the C-terminal part of Gpr1 were found to be detrimental for acetate acid sensitivity [11]. In the methanogen archaeon M. acetivorans by quantitative transcription analysis, the gene MA4008 was found to be highly expressed in acetic acid-grown cells compared with methanol-grown cells. The expression of this gene was similar to ack (acetate kinase) and ack (acetyl-CoA phosphotransferase), two of the enzymes required for acetate utilization, suggesting a role of MA4008 in acetate uptake [12].

The YaaH protein of E. coli, responsible for naming the family to which it belongs, has its annotation exclusively based on homology assumptions. In the present paper we provide for the first time experimental evidence for the functional role of YaaH as an acetate–succinate transporter proposing a new nomenclature for this protein.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

All the bacterial strains and plasmids used in the present study are listed in Tables 1 and 2 respectively. All strains used are isogenic with the wild-type E. coli K12 derivative strain MG1693. The cultures were maintained on slants of LB medium. All strains were grown in minimal medium [34] [34 mM NaH₂PO₄, 64 mM KH₂PO₄, 20 mM (NH₄)₂SO₄, 1 μM FeSO₄, 0.1 mM MgSO₄ and 10 μM CaCl₂] in shake flasks at a 1:10 ratio at 37°C and 200 rev./min throughout the present study unless stated otherwise. Carbon sources were glucose (1%, w/v) or acetic acid [1.67, 8.3, 16.6, 33.3, 50, 66.7 and 83.3 mM (pH 6.0)] as given in the Figure legends. Growth medium was supplemented with thymine (50 μg/ml) and the following antibiotics when appropriate: ampicillin (100 μg/ml), chloramphenicol (50 μg/ml) and kanamycin (50 μg/ml).

For the growth experiments in acetic acid, E. coli strains were cultured overnight in minimal medium supplemented with glucose 1% (w/v), collected by centrifugation at 5000 g for 1 min, washed twice in minimal medium (carbon-source free) and diluted to an attenuation of 600 nm at 0.05 in 150 μl of minimal medium (pH 6), supplemented with 1.7, 8, 16, 33.3, 50.0, 66.7 or 83.3 mM acetic acid in a 96-well microplate. Growth was monitored for 36 h using a Spectramax plus 384 absorbance microplate reader (Molecular Devices).

Construction of E. coli mutants

The yaaH (BBC232) and actP (BBC233)-null mutants were constructed using the primer pairs dyaaH1/dyaaH2 and dactP/dactP respectively, and following the λ-recombinase method [35], with a few modifications, as described previously [36]. The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides +5 to +535 of the yaaH gene (gene ID 944792) and the kanamycin-resistance cassette of plasmid pKD4 replaces nucleotides +69 to +1596 of actP (gene ID 948575). The gene deletions were verified by colony PCR using the primer pair P1yaaH/P2yaaH for yaaH and kt/P3actP and emb282/P1actP for actP. All chromosomal mutations were subsequently transferred to a fresh genetic background (MG1693 strain) by P1 transduction. The same method was used for the construction of the double mutant yaaH/actP (BBC234), from the respective single mutants.

For construction of pSVA9 plasmid expressing yaaH, a PCR fragment containing the entire yaaH coding sequence was amplified from MG1693 chromosome using the primer pair P3yaaH and P5yaaH. The resultant PCR fragment was cleaved with XbaI and HindIII and ligated into the high-copy pUC18 plasmid (GenBank® accession number L09136) digested with the same enzymes. Plasmid pWSK29 (GenBank® accession number AF016889-1), expressing the yaaH gene under its own putative promoter signals (pSVA10), was constructed using the same strategy, but with the primer pair P4yaaH/P5yaaH. Correct clones were verified by colony PCR and YaaH sequencing. All primers were obtained from StabVida and are listed in Table 3.

HPLC measurements

Samples at specific time points of growth were analysed for their concentration in organic acids, namely succinic and acetic

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**Table 1** List of strains used in the present study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant markers/genotype</th>
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<tr>
<td>BBC232</td>
<td>MG1693 yaaH (ΔyaaH::Cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>The present study</td>
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<tr>
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<td>MG1693 actP (ΔactP::Kan&lt;sup&gt;2&lt;/sup&gt;)</td>
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<tr>
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<td>BBC234 transformed with pUC18</td>
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<td>BBC234 transformed with pJ164G</td>
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**Table 2** List of plasmids used in the present study

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<td>Template for mutants construction; carries chloramphenicol-resistance cassette</td>
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<td>orR&lt;sup&gt;®&lt;/sup&gt;/Amp&lt;sup&gt;®&lt;/sup&gt;</td>
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<tr>
<td>pKD46</td>
<td>Temperature-sensitive λ-recombinase expression plasmid</td>
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<td>pMB1&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Fermentas</td>
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<td>Low-copy plasmid, constitutive expression</td>
<td>pSC101&lt;sup&gt;®&lt;/sup&gt;</td>
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<td>The present study</td>
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acid, and in carbohydrates, namely glucose. Cellular density was measured at 600 nm on a Genesys 20 spectrophotometer (Thermo Spectronic) and 1.5 ml samples were collected and centrifuged for 5 min at 16 100 × g. The supernatants were filtered through 0.22 μm syringe filters and were monitored using a Nanodrop1000 spectrophotometer. Transcription of isolated RNA to cDNA was carried out in a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories). Amplification conditions were as follows: 95 °C with a heating rate of 0.5 °C/s for 30 s. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program consisted of temperatures between 65 °C and 95 °C with a heating rate of 0.5 °C/s and a continuous fluorescence measurement. Additionally, the amplicons’ expected size and the absence of non-specific products were confirmed by analysis of the RT–PCR products in 1% agarose gels in 1× TAE (Tris/acetate/EDTA), stained with Midori Green and visualized under UV light. A negative control without template was conducted for each gene in each PCR run, and a control for DNA contamination was implemented by using the purified RNA samples as template. A positive control with the target cloned in an expression plasmid was conducted for each gene in each PCR run, and qPCRs were performed in triplicate for each cDNA sample tested. Threshold cycle (Ct) values were calculated by using Bio-Rad Laboratories CFX Manager software, and fold changes were calculated using the 2^(-ΔΔCt) method. Each gene was normalized to the housekeeping gene 16S rRNA (E. coli). Gene expression analysis by RT-PCR (real time PCR) Cells were incubated in minimal medium supplemented with 1% (w/v) glucose or 1.67 mM (0.1%) to 83.3 mM (0.5%) acetic acid and 1×10^7 cultured bacterial cells were harvested by centrifugation at specific time points (as given in the Figure legends). For total RNA isolation and purification the GRS Total RNA Kit – Blood & Cultured Cells kit from GRISP Research Solutions was used according to the manufacturer’s instructions. Purity and concentration of total RNA was evaluated by measuring the attenuation at 260 and 280 nm using a NanoDrop1000 spectrophotometer. Transcription of isolated RNA to cDNA was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Relative quantitative RT-PCR of the cDNA samples was carried out in a CFX96 Touch™ Real-Time PCR Detection System from Bio-Rad Laboratories using KAPA SYBR® FAST qPCR (quantitative PCR) Master Mix with SYBR® Green I as the detection agent. The primers used to amplify the selected genes using qPCR were designed using Primer Blast [37,38] and are listed in Table 3. Reactions were set up in a total volume of 20 μl using 2 μl of cDNA (diluted to 10−1), 10 μl of KAPA SYBR® FAST qPCR master mix (Kapa Biosystems), nuclease-free water and 200 nM of each gene-specific primer (Table 3) and performed in the CFX96 Touch™ machine (Bio-Rad Laboratories). Amplification conditions were as follows: 95 °C for 3 min, 40 cycles at 95 °C for 3 s and 57.6 °C for 30 s. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program consisted of temperatures between 65 °C and 95 °C with a heating rate of 0.5 °C/s and a continuous fluorescence measurement. Additionally, the amplicons’ expected size and the absence of non-specific products were confirmed by analysis of the RT–PCR products in 1% agarose gels in 1× TAE (Tris/acetate/EDTA), stained with Midori Green and visualized under UV light. A negative control without template was conducted for each gene in each PCR run, and a control for DNA contamination was implemented by using the purified RNA samples as template. A positive control with the target cloned in an expression plasmid was conducted for each gene in each PCR run, and qPCRs were performed in triplicate for each cDNA sample tested. Threshold cycle (Ct) values were calculated by using Bio-Rad Laboratories CFX Manager software, and fold changes were calculated using the 2^(-ΔΔCt) method. Each gene was normalized to the housekeeping gene 16S rRNA (E. coli).
changes were calculated as $2^{-\Delta C_T}$ with inner normalization to the 16S rRNA housekeeping gene [39]. All samples were then compared with the expression levels of the mid-exponential (3 h) samples of growth in glucose and expressed as relative fold expression. S.D. of $\Delta C_T$ values for three biological triplicates were propagated to obtain S.D. values for each fold change value [40].

Construction of yaaH mutations by site-directed mutagenesis

*yaaH* mutations were constructed in the plasmid pSV A9, using the oligonucleotide-directed mutagenesis technique [41]. The mutagenesis was performed using the DNA polymerase KAPA HiFi™ (Kapa Biosystems) with proofreading activity as follows: 10 ng of the template plasmid pSV A9 was combined with 10 pmol of the forward oligonucleotides P6yaaHmut or P7yaaHmut and the respective complementary reverse oligonucleotides (Table 3) containing the desired substitution (L131V or A164G) and used in the following PCR: 5 min at 95°C followed by 25 cycles of 20 s at 98°C, 15 s at 65°C, 5 min at 72°C, and a final extension step of 5 min at 72°C. In order to destroy the parental strands, the PCR products were incubated with the restriction enzyme DpnI (Fermentas) for 2 h at 37°C. This mixture was then used to transform *E. coli* XL1-Blue cells and plasmid extraction was performed on several clones with the Gene Elute™ Plasmid miniprep Kit (Sigma) to obtain plasmids pL131V and pA164G. Mutations were confirmed by sequencing using appropriate oligonucleotides for both DNA strands. The genes containing the desired mutations were introduced into *E. coli* Δ*yaaH Δ*actP* cells made competent by the rubidium chloride method [42].

RESULTS

Distinct physiological roles for yaaH and *actP* on acetate uptake

Single and double mutants for the genes *yaaH* and *actP* were constructed in the strain *E. coli* MG1693, as described in the Materials and methods section. The uptake of [1-14C]acetate (0.5 mM (pH 6.0)) was assessed in all above mentioned strains aerobically grown on mineral medium containing glucose, as the sole carbon and energy source, collected in mid-exponential phase (Figure 1). A strong decrease in the transport activity of labelled acetate was found in the double mutant Δ*yaaH Δ*actP*, whereas for the respective single mutants only a partial reduction in activity was detected compared with the wild-type strain (100% of activity). These observations were indicative of separate roles for YaaH and ActP in acetate transport.

The uptake of acetate was restored when *yaaH* was expressed in trans in Δ*yaaH Δ*actP* cells either from a low- or a high-copy number plasmid (Figure 1). The level of activity produced from the low-copy number plasmid was very similar to the one found for the single Δ*actP* mutant, whereas for the high-copy number plasmid the uptake values were approximately 50% greater than the activity of the wild-type strain.

Energetics and kinetics of the YaaH transporter

The effect of pH on the acetate transport activity associated with YaaH expression was measured in *E. coli* Δ*yaaH Δ*actP* cells transformed with the high-copy plasmid not expressing (pUC18) or expressing (pSV A9) *yaaH*. Data were taken from cells growing exponentially in mineral medium with glucose as the sole carbon and energy source (Figure 2A). For the pH range analysed, the uptake was maximum at pH 6.0 in cells harbouring pSV A9, with a decrease in activity at more acidic or more alkaline pH values, whereas, in cells transformed with pUC18, this pH effect was not found.

The protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone), which collapses the proton motive force, lowered transport to almost negligible values at pH 6.0 in cells expressing *yaaH* (Figure 2B). The potassium ionophore valinomycin and the sodium ionophore monensin, which disrupt the membrane electrical potential ($\Delta \psi$), had no significant effect on acetate uptake. In *E. coli* Δ*yaaH Δ*actP* cells transformed with pUC18 no visible inhibitory effect was observed with the protonophores and ionophores tested.

The dependence of the transporter activity on $\Delta \psi$, together with the effect of the protonophore CCCP, suggest that YaaH behaves as a secondary active acetate–proton symporter, energetically dependent on the proton motive force.

The initial uptake rates of [1-14C]acetate as a function of acetate acid concentration in *E. coli* Δ*yaaH Δ*actP* cells transformed with the empty pUC18 plasmid were residual for acetate acid uptake (Figure 3). Cells of the Δ*actP* mutant were used to assess the kinetic parameters associated with YaaH activity revealing an apparent $K_m$ value of 1.24 ± 0.13 mM acetate acid and an apparent $V_{max}$ value of 8.72 ± 0.37 nmol of acetate acid/min per mg of protein (Figure 3). As expected when overexpressing *yaaH* in Δ*yaaH Δ*actP* cells, a higher acetate transport activity (apparent $V_{max}$ value of 14.49 ± 0.63 nmol of acetate acid/min per mg of protein) was found. All these findings support the involvement of YaaH protein as an acetate transporter in *E. coli*.

The physiological role of YaaH and ActP on acetate assimilation

Wild-type cells display ability to growth on acetate acid (pH 6.0) under a range of concentrations varying from 1.67 mM (0.01%) to 83.3 mM (0.5%) (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540585add.htm), with concomitant acetate acid consumption as well as measurable acetate acid uptake. On the basis of this evidence we wondered about the role of acetate transporters in the ability of cells to grow on acetate acid.
mentioned, at the concentration indicated and pH 6.0 for 1 min before adding the radiolabelled substrate. Each data point represents the mean ± S.D. for three independent experiments (n = 6).

Figure 2 Energetics of the YaaH transporter
Effect of pH and of CCCP, valinomycin and monensin on the uptake of 0.5 mM [1-14C] acetic acid in cells of E. coli MG1693 ΔyaaH ΔactP transformed with pSVA9 or pUC18 plasmids grown as indicated in the legend of Figure 1. (A) Transport activity was determined in cells buffered in potassium phosphate at the pH values indicated. (B) Cells were pre-incubated with the compounds mentioned, at the concentration indicated and pH 6.0 for 1 min before adding the radiolabelled substrate. Each data point represents the mean ± S.D. for three independent experiments (n = 6).

Figure 3 Acetic acid kinetics of the YaaH transporter
Eadie–Hofstee plots of the initial uptake rates of [1-14C] acetic acid (pH 6.0) in glucose-grown cells grown as indicated in the legend of Figure 1. E. coli strains used: •, ΔyaaH ΔactP transformed with the pSVA9 plasmid expressing the yaaH transporter; ▲, ΔactP transformed with the pUC18 multicopy plasmid; and ○, ΔyaaH ΔactP transformed with the pUC18 multicopy plasmid. Each data point represents the mean ± S.D. for three independent experiments (n = 6). Inset, direct plot of the data shown in the main Figure.

We have found that single and double mutants have a significant alteration in their ability to grow in acetic acid as the sole carbon and energy source at 66.7 mM (0.4 %). This phenotype was even more pronounced at 83.3 mM (0.5 %), where the double and the ΔactP mutants display a longer lag phase (Figure 4). The mRNA expression of yaaH and actP examined at three time points (6, 12 and 24 h) has a similar profile (Figure 4H), with yaaH presenting a lower relative fold expression when compared with actP. In general, the expression of both genes increased over time independently of the acetate acid concentration used for growth, with a maximum level detected at 24 h in cultures with 66.7 mM acetic acid. Overall, these results highlight the relevance of acetate transporters on acetic acid utilization.

Distinct physiological roles for distinct acetate transporters
The observation that both YaaH and ActP act independently in the transport of acetate raised the question about what their respective distinctive physiological role might be during aerobic growth on glucose. We evaluated the expression of both yaaH and actP in cells grown on 0.2, 0.4 and 1.0 % (w/v) glucose in the wild-type strain as well as the relative acetic acid uptake over time. At low (0.2 %) and intermediate (0.4 %) glucose concentrations yaaH expression was almost negligible (Supplementary Figure S2 at http://www.biochemj.org/bj/454/bj4540585add.htm), whereas actP had a peak following entry into stationary phase. Taking the expression pattern into account we can infer that at these concentrations of glucose the relative activity for acetate uptake is probably more associated with actP.

A distinct behaviour was found for cells grown at high (1 %) glucose concentration where both yaaH and actP are expressed. To access the distinct physiological role of each transporter we compared the behaviour of the single and double mutant strains with the wild-type. In the experiment described in Figure 5, the glucose consumption profiles over time (Figures 5A–5D, ▲) were very similar in all strains, with the following specific growth rates (h−1): wild-type, 0.15; ΔactP, 0.14; ΔyaaH, 0.14; and ΔyaaH ΔactP, 0.12. Regarding acetate accumulation in the extracellular medium (Figures 5A–5D, ▼) the levels detected were identical in all four strains. The four strains achieved the same final D600 after 13 h of growth (Figures 5A–5D, ▪); however, regarding the acetate transport activity a distinct profile was observed for each strain (Figures 5A–5D, bars). In the wild-type strain, two peaks of activity were detected (Figure 5A), which can be correlated with the behaviour of the single mutants. The first peak, between 4 and 6 h of growth, is mostly associated with YaaH activity (Figure 5B), whereas the second peak, between 8 and 12 h, correlates with the ActP permease (Figure 5C). The double mutant displays only a residual acetate uptake activity throughout the growth curve (Figure 5D). It is worth mentioning that the acetate uptake activity profile detected in the wild-type correlates well with the sum of the values found for each single mutant (Figure 5E).

In the wild-type strain the mRNA expression profile was similar for both genes (Figure 5F) where two main peaks are detected, one at 5 h (middle exponential growth phase) and another at 10 h (stationary growth phase). These peaks of expression correlate with the mutants’ acetate uptake profiles. This behaviour suggests the existence of an unknown regulatory system at the transcriptional and/or translational level connecting the expression of these two genes.

Overall, the differences found among the four strains led us to postulate distinct physiological roles played by the two
Figure 4  The physiological role of YaaH and ActP on acetate assimilation

Growth profiles of E. coli MG1693 (●) and isogenic ΔactP (△), ΔyaaH (□) and ΔyaaH ΔactP (☆) mutants in minimal medium with 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G) acetic acid (pH 6.0). (H) Relative fold expression of yaaH and actP genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 h, normalized to 16S with S.D. of ΔC\textsubscript{T} values propagated for each fold change value, as described in the Materials and methods section. Each data point represents the mean ± S.D. for three independent experiments (n = 6).
transports when cells are grown at high glucose concentrations, with YaaH being more active at the exponential growth phase and ActP being most active in the entry to stationary growth phase, as previously postulated by Gimenez et al. [2].

YaaH as a succinate transporter

The range of YaaH transporter substrates was determined in the strain E. coli ΔyaaH ΔactP transformed with the pSVA9 plasmid, by adding non-labelled substrates (30 mM) to the reaction mixture containing labelled acetic acid (0.3 mM, pH 6.0). Boric, oxalic, pyruvic, lactic, malic and citric acid had no inhibitory effect on the uptake of acetate (results not shown). However, an inhibitory effect, higher than 80%, was found for formic, propionic, benzoic, salicylic and butyric acid (all monocarboxylic acids), as well as the dicarboxylic succinic acid. As can be seen in Figure 6 all of these monocarboxylic acids behaved as non-competitive inhibitors of acetic acid uptake, possibly by affecting the binding site for acetic acid. A different result was found for succinic acid which behaved as a competitive inhibitor for acetate uptake by the YaaH transporter (Figure 6), where an alteration of the slope (K_m) of the plots is observed instead of an alteration of the V_max value. To confirm this observation the kinetic parameters for the initial uptake rates of [1,4-14C]succinic acid were determined in cells of the double mutant transformed with the pSVA9 plasmid (Figure 7), using as control the same strain transformed with the pUC18 plasmid. In both strains, labelled succinic acid exhibited Michaelis–Menten kinetics with the following kinetic parameters: apparent K_m value of 1.18 ± 0.10 mM succinic acid and apparent V_max value of 10.05 ± 0.34 nmol of succinic acid/min per mg of protein for the pSVA9 transformants; and apparent K_m value of 2.19 ± 0.33 mM succinic acid and apparent V_max value of 5.50 ± 0.43 nmol of succinic acid/min per mg of protein for the pUC18 transformants. These data suggests that YaaH plays a role as a succinic acid transporter.

To further elucidate the functional role of YaaH in succinate uptake, the concentration of succinic acid was measured in the extracellular medium from the experiments described in Figure 5. In all strains succinic acid appeared as a minor subproduct during
growth in glucose, but two distinct profiles were observed among the four strains studied (results not shown). A sole peak of 10 ± 0.5 mg/l succinic acid was detected in the samples collected at 10 h of growth both in the wild-type and ΔactP strains. However, in the strains ΔyaaH and ΔyaaH ΔactP 14 ± 0.7 and 16 ± 0.8 mg/l of succinic acid were found at 9 h and 10 h of growth respectively. The highest levels of succinic acid found for the ΔyaaH-deleted strains, both single and double mutants, are consistent with a decrease in succinate import owing to the absence of the YaaH protein.

Two single residue mutations change the specificity in YaaH

Two single-nucleotide mutations (655C>G, encoding L219V and 755C>G, encoding A252G) in the yeast Ady2 promote a change in substrate specificity of this transporter allowing an efficient utilization of lactic acid [9], therefore we asked whether these residues could behave as in its bacterial homologue. The equivalent residues were identified based on the alignment between YaaH and Ady2 using ClustalW2 [13] (Figure 8A) and the two mutants (L131V and A164G) were obtained by site-directed mutagenesis in the pSV A9 plasmid.

The YaaH alleles were tested for the uptake of labelled D,L-[U-14C]lactic acid (pH 5.0) (Figure 8B) and, as expected, both substitutions improved the affinity and capacity of the acid uptake. The kinetic parameters estimated from the plots shown in Figure 8(B) revealed an apparent $K_m$ value of 4.15 ± 0.59 mM lactic acid for the wild-type, 2.88 ± 0.45 mM lactic acid for the A164G mutant and the highest affinity detected for the L131V mutant with an apparent $K_m$ value of 1.97 ± 0.25 mM lactic acid. The transport capacity in both mutants was also increased, with the following apparent $V_{max}$ values (nmol of lactic acid/min per mg of protein): 10.15 ± 0.76 for pSV A9, 11.17 ± 0.82 for the A164G mutant and 14.25 ± 0.79 for the L131V mutant. In both mutants lactic acid uptake was competitively inhibited by acetic and succinic acid, showing that these acids are still accepted by the YaaH transporter (Figure 8C).

All of these assays were performed on glucose-grown cells, conditions where the E. coli l-lactate transporters [lldP (l-lactate permease) and glcA (glycolate permease)] are down-regulated, thus the level of lactic acid transport detected in cells transformed with the empty pUC18 plasmid (Figure 8B, ⊘) is only basal.
In summary, the results of the present study suggest that YaaH behaves as a secondary active acetate–proton symporter with an apparent \( K_m \) value of 1.24 ± 0.13 mM acetic acid and an apparent \( V_{max} \) value of 8.72 ± 0.37 nmol of acetic acid/min per mg of protein, at pH 6.0, energetically dependent on the protonotive force, the same as many carboxylate transporters found in microorganisms [2,17–20].

YaaH is a succinic acid transporter

In contrast with what has been described for other family members [4,5], the YaaH transporter in \textit{E. coli} seems to be highly specific for acetic acid (a monocarboxylic acid) and for succinic acid (a dicarboxylic acid). It has been reported that \textit{E. coli} overexpressed membrane proteins have to be accommodated at the plasma membrane, limiting the level of maximum activity.

The basal level of acetate uptake found in the double mutant \( \Delta yaaH, \Delta actP \) (Figure 1) can be attributed either to the existence of other transporters or to the simple diffusion of the undissoicated form of the acid across the plasma membrane. It is commonly accepted that the undissoicated form of the acid enters bacteria mostly by diffusion [29], whereas the dissociated form needs assistance from acetate carrier systems [45]. As our assays were carried out at pH 6.0, and taking into account the pK\(_{a}\) value of acetic acid (4.74), only 5.44% of the acid is in its lipid-soluble (uncharged) form. Since the acid is mostly in the dissociated form, passive diffusion is limited by its solubility and permeability [29,30,46].

Acetate uptake in glucose-grown cells of the double mutant \( \Delta yaaH, \Delta actP \) follows saturable kinetics in pH ranging from 5.0 to 7.0 (results not shown) with similar kinetic parameters found for \( \Delta yaaH, \Delta actP \) transformed with pUC18 (Figure 3).

Overall, our data suggest that the existence of other as yet unidentified acetate transporter(s) in \textit{E. coli} cannot be ruled out.

The YaaH acetate uptake activity was optimal at pH 6.0 with decreasing activity both at more acidic and alkaline pH values. This observation further strengthens our hypothesis about the minor role that passive diffusion plays on acetate influx under the conditions analysed. If the undissoicated acid was permeating the membrane mostly by simple diffusion, decreasing the pH would increase the acid influx owing to an increase in the relative amount of the undissoicated acid, but this increase in uptake was not observed. Furthermore, the inhibition of acetate transport by the uncoupler CCCP in the \( \Delta yaaH, \Delta actP \) strain transformed with pSVA9 indicates that the driving force used by YaaH is the transmembrane electrochemical potential. The fact that this uncoupler had no effect on \( \Delta yaaH, \Delta actP \) cells carrying pUC18 demonstrates that CCCP, under the conditions and for the incubation times tested, had no other pleotropic effects [15,16].

In summary, the results of the present study suggest that YaaH/SatP (YaaH), an \textit{E. coli} succinate–acetate transporter

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Site-directed mutagenesis of the yaaH gene
\textbf{A} Predicted transmembrane segments V and VI of YaaH (http://www.cbs.dtu.dk/services/TMHMM) aligned with Ady2 using ClustalW2. The conserved residues are marked with an asterisk (*) and the residues mutated in the present study are highlighted in black boxes.
\textbf{B} Initial uptake rates of \( [\text{U}^{-1}\text{C}]\)lactic acid (pH 5.0) as a function of the acid concentration.
\textbf{C} Initial uptake rates of \( [\text{U}^{-1}\text{C}]\)lactic acid (pH 5.0) as a function of the acid concentration indicated in each plot in cells transformed with pA164G (\textbf{D}) Eadie–Hofstee plot of the initial uptake rates of \( [\text{U}^{-1}\text{C}]\)lactic acid (pH 5.0) in the absence (\textbf{I}) and in the presence (\textbf{II}, \textbf{II}, \textbf{II}, \textbf{II}) of non-labelled acetic and succinic acid, at the concentration indicated in each plot in cells transformed with pUC18 (\textbf{II}). Cells were grown and collected as described in the legend of Figure 1. Each data point represents the mean ± S.D. for three independent experiments (\( n = 6 \)).
\textbf{DISCUSSION}

YaaH is an acetate–proton symporter

In the present study we demonstrate that acetate transport in \textit{E. coli} occurs via, at least, two distinct proteins: ActP, a member of the Sodium:Solute Symporter Family (Transporter Classification Database TCDB 2.2.21), and YaaH, a member of the YaaH family (Transporter Classification Database TCID 2.9.6). Our data fully agree with the previous work of Wagner et al. [14] reporting the presence of two separate processes for acetate transport that, to our knowledge, are, most probably, associated with the AcetP and YaaH proteins.

The single mutants of the genes \textit{yaaH} and \textit{actP} revealed a partial reduction in acetate uptake when compared with the wild-type strain, whereas the double mutant displays a significant reduction, indicative of separate roles for YaaH and ActP in acetate transport activity. Additionally, the level of acetate uptake activity in \( \Delta yaaH, \Delta actP \) cells, expressing the \textit{yaaH} gene in trans, fully agrees with its role as a transporter. When \textit{yaaH} is expressed in a low-copy number plasmid, a lower apparent \( V_{max} \) value was found when compared with the one observed for the wild-type since the \textit{actP} gene is missing. However, as expected, when using the high-copy number plasmid there was an increase in the acetate uptake, associated with the overexpression of the YaaH protein. A 50% increase in activity is quite significant since overexpressed membrane proteins have to be accommodated at the plasma membrane, limiting the level of maximum activity.

The basal level of acetate uptake found in the double mutant \( \Delta yaaH, \Delta actP \) (Figure 1) can be attributed either to the existence of other transporters or to the simple diffusion of the undissoicated form of the acid across the plasma membrane. It is commonly accepted that the undissoicated form of the acid enters bacteria mostly by diffusion [29], whereas the dissociated form needs assistance from acetate carrier systems [45]. As our assays were carried out at pH 6.0, and taking into account the pK\(_{a}\) value of acetic acid (4.74), only 5.44% of the acid is in its lipid-soluble (uncharged) form. Since the acid is mostly in the dissociated form, passive diffusion is limited by its solubility and permeability [29,30,46]. Acetate uptake in glucose-grown cells of the double mutant \( \Delta yaaH, \Delta actP \) follows saturable kinetics in pH ranging from 5.0 to 7.0 (results not shown) with similar kinetic parameters found for \( \Delta yaaH, \Delta actP \) transformed with pUC18 (Figure 3).

Overall, our data suggest that the existence of other as yet unidentified acetate transporter(s) in \textit{E. coli} cannot be ruled out.

The YaaH acetate uptake activity was optimal at pH 6.0 with decreasing activity both at more acidic and alkaline pH values. This observation further strengthens our hypothesis about the minor role that passive diffusion plays on acetate influx under the conditions analysed. If the undissoicated acid was permeating the membrane mostly by simple diffusion, decreasing the pH would increase the acid influx owing to an increase in the relative amount of the undissoicated acid, but this increase in uptake was not observed. Furthermore, the inhibition of acetate transport by the uncoupler CCCP in the \( \Delta yaaH, \Delta actP \) strain transformed with pSVA9 indicates that the driving force used by YaaH is the transmembrane electrochemical potential. The fact that this uncoupler had no effect on \( \Delta yaaH, \Delta actP \) cells carrying pUC18 demonstrates that CCCP, under the conditions and for the incubation times tested, had no other pleotropic effects [15,16].

In summary, the results of the present study suggest that YaaH behaves as a secondary active acetate–proton symporter with an apparent \( K_m \) value of 1.24 ± 0.13 mM acetic acid and an apparent \( V_{max} \) value of 8.72 ± 0.37 nmol of acetic acid/min per mg of protein, at pH 6.0, energetically dependent on the protonotive force, the same as many carboxylate transporters found in microorganisms [2,17–20].

YaaH is a succinic acid transporter

In contrast with what has been described for other family members [4,5], the YaaH transporter in \textit{E. coli} seems to be highly specific for acetic acid (a monocarboxylic acid) and for succinic acid (a dicarboxylic acid). It has been reported that \textit{E. coli} cells lacking the known aerobic and anaerobic \( C_4 \)-dicarboxylate carriers (DctA, DcuA, DcuB, DcuC and DcuD or CitT) still display ability to aerobically grow on succinate at pH 6.0, by expressing a different succinic acid carrier [21]. The authors mentioned that such a carrier was not specific for \( C_4 \)-dicarboxylates and also had a low
affinity for monocarboxylates, such as propionate, butyrate and acetate. A recent study in *E. coli* reported that in cells grown aerobically on succinic acid two succinate transporters DctA and DauA coexist, with DauA as the lower affinity and lower capacity transporter [22]. The authors demonstrated that DauA is essential for the expression and activity of DctA, with DctA as the main transporter at pH 7.0 and DauA at pH 5.0. The mutants ΔdauA at pH 5.0 and ΔdctA at pH 7.0, grown in succinate, display residual succinic acid transport activity pointing to the presence of a very low affinity uncharacterized transporter [22]. Our finding that YaaH is able to transport succinic acid with a lower apparent *K*ₐ value (1.18 ± 0.10 mM succinic acid at pH 6.0) compared with the ones found for the above mentioned transporters, can contribute to fill this gap. Furthermore, YaaH only recognizes succinate, and does not accept other C₄-dicarboxylates, a feature that is in good agreement with the literature [21].

Taking into account the role of the YaaH protein in acetic and succinic acid uptake, we propose a new nomenclature for this permease, SatP, a Succinate–Acetate Transporter Protein.

**L131V and A164G change the specificity of YaaH**

The yeast homologue of YaaH is Ady2, a monocarboxylate–proton symporter, shared by acetate, propionate, lactate and formate, whose expression is repressed by glucose [3,5,8]. By direct evolution for growth in lactic acid two mutants L219V and A252G were identified in Ady2 [9]. We wondered whether the corresponding mutations in YaaH, L131V and A164G (Figure 8A), would lead to a gain of function for lactic acid uptake. It is known that the uptake of lactate in *E. coli*, L131V and A164G (Figure 8A), would lead to a gain of function for lactic acid uptake. It is known that the uptake of lactate in *E. coli* is mediated by two distinct proton symporters, *lldP* and *glaA* [18]. These transporters are repressed in glucose [23], thus only a basal level of lactic acid uptake was detected under the conditions used in the present study for the control strain (ΔyaaH ΔactP transformed with pUC18). Glucose-grown cells of ΔyaaH ΔactP expressing the yaaH mutants L131V and A164G display an enhanced affinity and capacity for lactic acid revealing an acquired ability to transport this substrate compared with the wild-type. Despite the distinct range of substrates of YaaH and Ady2 they share common molecular traits regarding the substrate-binding site since mutations in conserved amino acid residues lead to similar physiological behaviour, as we have shown for the gain of function for lactic acid uptake.

**YaaH and the acetate switch**

In the present study we addressed the physiological relevance of the existence of two acetate carriers ActP and YaaH by following their activity along aerobic growth in glucose minimal medium. When *E. coli* is grown aerobically in glucose as the carbon source a rapid growth phase is observed, with the consumption of the sugar and the excretion of acetate. This is followed by a slower growth phase with a switch to the use of the excreted acetate as a source of carbon and energy [1]. The formation of acetate depends primarily on the PTA–ACKA (acetate kinase) pathway with PTA reversibly converting acetyl-CoA and inorganic phosphate into acetyl~P and coenzyme A, whereas ACKA reversibly converts acetyl~P and ADP into acetate and ATP (for a review see [1]). YaaH functions primarily during the exponential phase of growth prior to the acetate switch.

When the carbon flux exceeds the capacity of the central metabolic pathways, acetate is assimilated by *acs*, which activates acetate to acetyl-CoA with the concomitant conversion of ATP into AMP and pyrophosphate [24,25]. Acetyl-CoA can be used in the tricarboxylic acid cycle via the glyoxylate bypass [26]. ActP seems to be involved in this step being responsible for the reutilization of acetate, scavenging micromolar concentrations of this compound [2]. This idea was reinforced in the present study by showing a more active role of this permease at the stationary phase after the acetate switch. Furthermore, previous work has shown that transcription of the *acs-yjcH-actP* operon is low in the presence of glucose excess due to low cAMP levels that regulate this operon [27] and that *acs* transcription increases with extracellular acetate formation [25]. This is in accordance with what was observed for *actP* expression that increased at the entry into stationary phase.

**YaaH in acetate assimilation**

We have shown that cells’ ability to assimilate acetic acid is not strictly dependent on either YaaH or ActP activity. As shown in Figure 4, wild-type and deleted mutant strains were able to grow over a range of concentrations varying from 1.67 to 83.3 mM acetic acid, as sole carbon and energy source at pH 6.0. Both YaaH and ActP display a functional role at the highest concentrations, followed by an increased level of expression, with a peak for both genes at 66.7 mM acetic acid. This is evidence for the role of these transporters in acetate assimilation allowing cells to better control the uptake of the acid, in particular at very high concentrations.

Acetate-metabolizing cultures of *E. coli* are particularly relevant for the biotechnology industry since the accumulation of acetate in the extracellular medium poses an obstacle to high cell density cultivation and protein production [28,47]. Acetate toxicity and growth inhibition can be attributed to an acidification of the cytoplasm. It has been assumed that acetate excretion into the environment is a problem due to the lipophilic nature of the undissociated form that can permeate membranes, uncoupling the transmembrane pH gradient and becoming highly toxic [29–32]. However, acetate seems to have only a small effect on the decrease of the intracellular pH and therefore the growth inhibition is possibly not only correlated to the acidification of the intracellular medium [30], but also to a complex result of the anion balance of the cell [33]. Although not crucial for cells to grow on glucose or acetic acid, YaaH and ActP contribute to the acetate–succinate intracellular balance. Acetate transporters might be future targets for improving existing protein expression systems as *E. coli* is one of the most important micro-organisms for biotechnological applications where acetate represents a major unwanted by-product.

**AUTHOR CONTRIBUTION**

Joana Sá-Pessoa performed experiments; Joana Sá-Pessoa, David Ribas, Inês Jesus Silva and Sandra Cristina Viegas, constructed the strains and plasmids; Joana Sá-Pessoa and Margarida Casal designed the experiments and analysed the data; Sandra Paiva, Cecilia Maria Arraiano and Margarida Casal supervised and co-ordinated the experiments; and Joana Sá-Pessoa and Margarida Casal wrote the paper.

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SUPPLEMENTARY ONLINE DATA
SATP (YaaH), a succinate–acetate transporter protein in *Escherichia coli*

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Figure S1  Acetate uptake profiles of cells grown on acetic acid

Growth profiles of *E. coli* MG1693 cells grown on minimal medium with 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G) acetic acid (pH 6.0). ■, Δ*, OD600; ▲, acetic acid (g/l); bars, transport activity measured with 0.5 mM [14C]acetic acid (pH 6.0). Error bars represent the S.D.

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Figure S2  Acetate uptake profiles of cells grown on glucose

Growth profiles of E. coli MG1693 cells grown on minimal medium with 0.2 % (A) and 0.4 % (B) glucose. ■, OD₆₀₀; ▲, glucose (g/l); ▼, acetic acid (g/l); bars, transport activity measured with 0.5 mM [¹⁴C]acetic acid (pH 6.0). (C) Relative fold expression of yaaH and actP genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 h of growth, normalized to 16S with S.D. of ΔC₅ values propagated for each fold change value, as described in the Materials and methods section of the main text. Each data point represents the mean ± S.D. for three independent experiments (n = 6).

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