The Bacillus subtilis ywkA gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate

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A transcriptome comparison of a wild-type Bacillus subtilis strain growing under glycolytic or gluconeogenic conditions was performed. In particular, it revealed that the ywkA gene, one of the four paralogues putatively encoding a malic enzyme, was more transcribed during gluconeogenesis. Using a lacZ reporter fusion to the ywkA promoter, it was shown that ywkA was specifically induced by external malate and not subject to glucose catabolite repression. Northern analysis confirmed this expression pattern and demonstrated that ywkA is cotranscribed with the downstream ywkB gene. The ywkA gene product was purified and biochemical studies demonstrated its malic enzyme activity, which was 10-fold higher with NAD than with NADP (kcat/Km 102 and 10 s−1 mM−1, respectively). However, physiological tests with single and multiple mutant strains affected in ywkA and/or in ywkA paralogues showed that ywkA does not contribute to efficient utilization of malate for growth. Transposon mutagenesis allowed the identification of the uncharacterized YufL/YufM two-component system as being responsible for the control of ywkA expression. Genetic analysis and in vitro studies with purified YufM protein showed that YufM binds just upstream of ywkA promoter and activates ywkA transcription in response to the presence of malate in the extracellular medium, transmitted by YufL. ywkA and yufL/yufM could thus be renamed maeA for malic enzyme and malK/malR for malate kinase sensor/malate response regulator, respectively.

INTRODUCTION

Malic enzymes catalyse the reversible oxidative decarboxylation of malate into pyruvate with reduction of NAD or NADP. In eukaryotes, several isoforms of this enzyme have been characterized. Three classes of malic enzymes have been defined on the basis of their coenzyme specificity and their capability to also decarboxylate oxaloacetate (Kobayashi et al., 1989). A first class of NADP-dependent malic enzymes (EC 1.1.1.40), decarboxylating malate and also oxaloacetate, are found in cytosol, chloroplasts and mitochondria; a second group of enzymes, preferentially utilizing NAD (EC 1.1.1.38), are also capable of decarboxylating oxaloacetate and are found in bacteria and insects; finally, a third class of NAD-dependent malic enzymes (EC 1.1.1.39), unable to use oxaloacetate, have been found only in mitochondrial matrix. In prokaryotes, malic enzymes are also widely distributed (Murai et al., 1971; Diesterhaft & Freese, 1973; Lamed & Zeikus, 1981; Knichel & Radler, 1982; Bartolucci et al., 1987; Kobayashi et al., 1989; Kawai et al., 1996; Voegele et al., 1999). However, few of them have been characterized so far, and in particular, few reports allowed distinction of whether the observed malic enzyme activity resulted from one or several isozymes. In Escherichia coli, both NAD- and NADP-dependent malic enzymes, named SfC and B2463 respectively, have been characterized (Murai et al., 1971; Hansen & Juni, 1974, 1975; Stols & Donnelly, 1997) but their respective physiological functions have not been precisely documented. It has been suggested that SfC is involved in gluconeogenesis by providing pyruvate, then converted into

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Abbreviations: TCA, tricarboxylic acid; TCS, two-component system.

The online version of this paper (at http://mic.sgmjournals.org) contains a table listing the oligonucleotides used in this study and a figure showing the overproduction and purification of His6-YufM.
phosphoenolpyruvate by a phosphoenolpyruvate synthase (Hansen & Juni, 1975), and that B2463 is involved in the supply of NADPH and acetyl-CoA, necessary for anabolic reactions during growth on C₄-dicarboxylates as sole carbon sources (Murai et al., 1971; Hansen & Juni, 1974, 1975). The synthesis of both enzymes is repressed in the presence of glucose while the expression of sfCA, encoding the NAD-dependent malic enzyme, is inducible by malate (Murai et al., 1971). The combined deletion of the two genes leads to a severe growth defect only on C₄-dicarboxylates, thus indicating that malic enzyme activity is necessary under such growth conditions in E. coli (van der Rest et al., 2000). Rhizobium meliloti, a nitrogen-fixing Gram-negative symbiont of alfalfa, also synthesizes two malic enzymes named DME and TME, which are NAD- and NADP-dependent, respectively (Driscoll & Finan, 1997; Mitsch et al., 1998). The former has been shown to be involved in symbiotic nitrogen fixation as a result of its role for growth in the nodule, where C₄-dicarboxylates are the major carbon and energy source provided by the host (Driscoll & Finan, 1993; Dunn, 1998), while the latter has been proposed to function as a generator of NADPH necessary for biosynthesis reactions (Driscoll & Finan, 1996).

Interestingly, the analysis of the Bacillus subtilis complete genome sequence revealed the presence of four paralogous genes encoding putative malic enzymes: ywkA, malS, ytsJ and mleA. None of these genes has been previously characterized. Indeed, little information has been reported about malic enzyme activity in this bacterial species. Diesterhaft & Freese (1973) demonstrated that the presence of malate stimulates the synthesis of an NADP/NAD-dependent malic enzyme activity.

Amino acid sequence comparisons indicate that the ywkA and malS products, on the one hand, and the ytsJ and mleA products, on the other hand, belong to two distinct subgroups (60% identity between YwkA and MalS; 40% identity between YtsJ and MleA; but 28% identity between YtsJ and MalS). Furthermore, YwkA and MalS share 40% identity with SfCA, an NAD-dependent malic enzyme which was found to be more similar to malolactic enzymes than to other malic enzymes (Groisillier & Lonvaud-Funel, 1999). However, malic and malolactic enzymes are difficult to distinguish on the basis of the amino acid sequence because they are highly similar and harbour common conserved sequence motifs (Denayrolles et al., 1994; Kawai et al., 1996; Groisillier & Lonvaud-Funel, 1999) and because only very few malolactic enzymes have been enzymically characterized (Ansanay et al., 1993; Arthers & Lloyd, 1999). Recently, a function for mleA in B. subtilis has been proposed by Wei et al. (2000). mleA is the second gene of a bicistronic operon including the mleN gene. mleN has been demonstrated to encode an antiporter that achieves simultaneous Na⁺/H⁺ and malate/lactate antiport. Therefore, MleA is strongly predicted to be a malolactic enzyme.

The examination of the sequence of the B. subtilis chromosome indicates that ywkA is probably cotranscribed with ywkB, which encodes a protein of unknown function. malS is linked to ytsP, an unknown gene, and ytsJ is linked to dnaE, which encodes the second essential DNA polymerase (Dervyn et al., 2001). Thus, the genetic organization of these three genes did not give any precise clue about their possible physiological roles.

Bacteria have to adapt to frequent changes in nutritional availability and, more generally, in environmental conditions. This is particularly true for free-living soil bacteria like B. subtilis. To accommodate gene expression to environmental conditions, two-component sensor–regulator systems are frequently used by bacteria. The two-component systems (TCSs) are composed of sensory protein kinases and of response regulator proteins. The protein kinases are typically located in the cytoplasmic membrane and comprise an N-terminal sensory and a conserved C-terminal transmitter domain. Binding of the signal ligand to the sensory domain induces ATP hydrolysis and autophosphorylation of the transmitter domain. The phosphoryl group is subsequently transferred to the cognate response regulator, leading to the activation of its regulatory property, most commonly transcriptional activation. More than 30 TCSs can be predicted from the complete B. subtilis genome sequence. However, a precise adaptive role has been identified for only a few of them (Perego & Hoch, 2002). Among them, two, YdbFG (DctsR) (Asai et al., 2000) and CitST (Yamamoto et al., 2000), appeared to be able to sense the presence of tricarboxylic acid (TCA) cycle intermediates in the medium and to activate the expression of genes encoding transporters for these compounds. Furthermore, a comprehensive DNA microarray analysis of the B. subtilis TCSs has suggested that ywkA could be a target of YufL/YufM (Kobayashi et al., 2001).

The starting point of the study reported here is a global transcriptome comparison of a wild-type B. subtilis strain growing in minimal medium containing glucose or malate as sole carbon source. Among the genes which appeared to be more transcribed on malate than on glucose was ywkA, but not malS or ytsJ. The regulation of the expression of the ywkA gene has therefore been further investigated, and the uncharacterized YufL/YufM TCS has been demonstrated to control directly ywkA transcription in response to the presence of malate. The enzymic activity encoded by ywkA has been characterized. Finally, single and multiple mutant strains for ywkA and the other putative malic enzyme genes have been constructed and studied to investigate the physiological role of YwkA.

METHODS

Bacterial strains and growth conditions. E. coli TG1 [K12 Δ(lac–pro) supE thi hsd–S–F’ traD36 proA+B– lacIq lacZAM15] was used as a general cloning host and E. coli strain M15 pREP4 (Qiagen) was used for overproduction of His–YufM and His–YwkA. E. coli strains were grown in Luria–Bertani broth (LB) supplemented with antibiotics when necessary (ampicillin, 100 μg mL⁻¹;
kanamycin, 25 mg L⁻¹). Conventional calcium-shock or electro-
poration procedures were used for transformation (Sambrook
et al., 1989).

The B. subtilis strains used in this work are listed in Table 1. Standard
procedures were used to transform B. subtilis (Anagnostopoulos &
Spizizen, 1961). B. subtilis strains were grown in LB, except when stated
otherwise. Antibiotics for selection were added at 5 mg L⁻¹ (chloro-
amphenicol), 0-4 mg L⁻¹ (erythromycin), 100 mg L⁻¹ (spectomycin-
ycin), or 5 mg L⁻¹ (kanamycin). All cultures were performed at 37 °C.
Growth tests were performed in MM minimal medium [K₂HPO₄
85 mM, KH₂PO₄ 40 mM, (NH₄)₂SO₄ 15 mM, trisodium citrate
6 mM, MgSO₄ 0-8 mM, pH 7] supplemented with tryptophan
(0-005 %) and carbon sources (0-5 %). Cultures for β-galactosidase
assays were performed in CQTHC minimal medium [C minimal
medium (Aymerich et al., 1986) supplemented with 0-005 % try-
ptophan, 0-05 % glutamine, 0-05 % casein hydrolysate] supplemented
with 1 % glucose, 1 % glycerol, 1 % fumarate, 1 % malate, or 1 %
succinate plus 1 % glutamate.

**DNA manipulation.** PCR products were purified with the QIAquick
PCR purification kit (Qiagen). Plasmid DNA was extracted and
purified from E. coli with the QIAprep spin miniprep kit (Qiagen).
Restriction enzymes and T4 DNA ligase were used as recommended
by the manufacturers. Pyrobest DNA polymerase (Takara) was used
in PCR reactions with primer pairs EagI-ytnP/XbaI-malS and BamHI-ytzB/EcoRI-ytzB
and one to the 500 bp downstream of the
malS ORF, were generated
with primer pairs Eagl-ytnP/XbaI-malS and BamHI-ytzB/EcoRI-ytzB
(see supplementary table), respectively. The two PCR fragments were
then ligated together with the spectinomycin cassette flanked by the
BamHI and XbaI restriction sites into vector pJH101 (Ferrari et al.,
1982) to generate plasmid pTD110, which was used to transform
B. subtilis.

**RNA isolation and Northern blot analysis.** For RNA preparation,
cell pellets were obtained from a mid-exponential-phase (OD₆₀₀ 0-8)
culture in S6 medium (Fujita & Freese, 1981) containing 25 mM
malate or 37-5 mM malate. Total RNAs were isolated from cells
essentially as previously described (Yoshida et al., 2001). For Northern
blot analysis, RNA samples (2 µg each) were electrophoresed in

**Table 1. B. subtilis strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>168CA</td>
<td>trpC2</td>
<td>Lab. stock</td>
</tr>
<tr>
<td>PS1716</td>
<td>amyE'::PywkA'::lacZ-cat</td>
<td>pPS61→168CA</td>
</tr>
<tr>
<td>YWKAd</td>
<td>ywkA'::pMUTINT3(ery)</td>
<td>JFAN*</td>
</tr>
<tr>
<td>BFA91</td>
<td>yrsf'::pMUTIN2</td>
<td>BFA† collection</td>
</tr>
<tr>
<td>GTD102</td>
<td>ywkA'::kan</td>
<td>pEC23→YWKAd</td>
</tr>
<tr>
<td>GTD110</td>
<td>ΔmalS::sp</td>
<td>This study</td>
</tr>
<tr>
<td>GTD111</td>
<td>ywkA'::kan ΔmalS::sp</td>
<td>GTD102 DNA × GTD110</td>
</tr>
<tr>
<td>GTD121</td>
<td>yrsf'::pMUTIN2 ywkA'::kan</td>
<td>BFA91 DNA × GTD102</td>
</tr>
<tr>
<td>GTD122</td>
<td>yrsf'::pMUTIN2 ΔmalS::sp</td>
<td>BFA91 DNA × GTD110</td>
</tr>
<tr>
<td>GTD123</td>
<td>yrsf'::pMUTIN2 ywkA'::kan ΔmalS::sp</td>
<td>BFA91 DNA × GTD111</td>
</tr>
<tr>
<td>1A250</td>
<td>alsR1 ivalA</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>1A147</td>
<td>ccpA1 alsR1 ivalA</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>C1</td>
<td>yufL'::Tn10 amyE'::PywkA'::lacZ-cat</td>
<td>This study</td>
</tr>
<tr>
<td>BFA1431</td>
<td>yufL'::pMUTIN2</td>
<td>BFA collection</td>
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<td>pEC23→BFA1431</td>
</tr>
<tr>
<td>GTD134</td>
<td>yufL'::p[MUTIN2 Δ(lacZ-ery)::pEC23(ks)]amyE'::PywkA'::lacZ-cat</td>
<td>GTD133 DNA × PS1716</td>
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<tr>
<td>BFA1432</td>
<td>yufM'::pMUTIN2</td>
<td>This study</td>
</tr>
<tr>
<td>GTD131</td>
<td>yufM'::p[MUTIN2 Δ(lacZ-ery)::pEC23(ks)]</td>
<td>pEC23→BFA1432</td>
</tr>
<tr>
<td>GTD132</td>
<td>yufM'::p[MUTIN2 Δ(lacZ-ery)::pEC23(ks)]amyE'::PywkA'::lacZ-cat</td>
<td>GTD131 DNA × PS1716</td>
</tr>
</tbody>
</table>

*Japan Function Analysis Network for B. subtilis. Plasmid pMUTINT3 used to construct strain YWKAd is a derivative of the pMUTIN plasmid
(Moriya et al., 1998).†Bacillus Subtilis Functional Analysis programme.
glyoxal gels, transferred to a Hybond-N membrane (Amersham) and hybridized with a labelled probe as described previously (Yoshida et al., 1997). To prepare the probes for ywkA, ywkB and gapB, parts of their coding regions were amplified by PCR using chromosomal DNA of *B. subtilis* 168 as a template and primer pairs ywkA-N1 and ywkA-N2, ywkB-N1 and ywkB-N2, and gapB-N1 and gapB-N2, respectively (see supplementary table). The PCR products were labelled radioactively by using the *Bca* BEST labelling kit (Takara Shuzo) and [32P]ATP (ICN Biomedicals).

**Preparation of fluorescently labelled cDNA, hybridization and microarray analysis.** The fluorescently labelled cDNA probes used for hybridization to DNA microarrays were prepared by a two-step procedure, as described previously (Ogura et al., 2001).

The microarrays that we used in this study contained 4055 protein genes, 45 not being spotted due to a problem with DNA amplification by PCR, as well as 39 calf thymus DNA spots as negative controls. The hybridization and microarray analysis were performed as described previously (Ogura et al., 2001; Yoshida et al., 2001). The mean Cy3 and Cy5 fluorescence intensities for each spot were calculated, the background being taken as the mean of the intensities of the 39 calf thymus DNA spots. After subtracting the background from all the intensities of the *B. subtilis* gene spots, and their normalization using the total Cy3 and Cy5 intensities, we calculated the expression ratios. To get reliable ratios, we ignored the spots of the intensities used as numerators for this calculation which were less than the background, and replaced the intensities used as denominators with the standard deviation of the mean intensity of the negative controls if they were lower than them.

**Primer extension.** To map the 5'-end of the *ywkA* transcript by primer extension, 50 μg of each RNA was annealed to primer rev (see supplementary table) that had been labelled at its 5'-end by a MEGALABEL kit (Takara Shuzo) and [32P]ATP (Amersham). Primer extension reactions were carried out as described previously (Yoshida et al., 1997).

**Construction of the mini-Tn10 transposition library and genetic screening.** The mini-Tn10 delivery vector pIC333 (Steinmetz & Richter, 1994) was used for transposon mutagenesis. Outside the transposon, this plasmid carries a thermosensitive Gram-positive origin of replication, an erythromycin-resistance gene, and a gene encoding a modified transposase with relaxed target specificity. The mini-transposon itself confers spectinomycin resistance and contains the pUC origin of replication in *E. coli*. pIC333 was introduced into PS176 at 25°C using erythromycin selection. Single transformant colonies were used to inoculate 12 independent cultures in 2 ml LB containing spectinomycin. At the beginning of the exponential growth phase, the temperature was shifted from 25 to 37°C and cells were grown for 4 h more at 37°C. Dilutions of the cultures were spread on plates of MM plus cascin hydrolysate (0-05%) or LB medium, plus X-Gal supplemented with glucose (0-5%) or maltate (0-5%) to screen for clones with deregulated *ywkA*: -lacZ expression.

**β-Galactosidase assays.** β-Galactosidase activities were measured using the method of Miller (1972) on cell extracts prepared by lysozyme treatment and centrifugation. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay solution. One unit of β-galactosidase activity is defined as the amount of enzyme that produces 1 nmol ONPG min⁻¹ at 20°C.

**Expression and purification of His<sub>6</sub>-YufM.** His<sub>6</sub>-YufM was overexpressed and purified using the QiAexpress kit (Qiagen). *yufM* coding sequence (705 bp) was integrated into *E. coli* expression vector pQE-30 using primers BamHI-YufM and PstI-YufM (see supplementary table) in order to produce a fusion protein with a hexahistidine tag at the N-terminus. The resulting plasmid, pTPd3, was transformed into *E. coli* M15 pREP4 groESL to generate the strain used for production, GTDp3. All the subsequent procedures were performed as described in the QiAexpress manual. His<sub>6</sub>-YufM was stored in a buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM DTT and 0-5 mM EDTA.

**Expression and purification of His<sub>6</sub>-YwkA.** His<sub>6</sub>-YwkA was overexpressed and purified as described for His<sub>6</sub>-YufM. Thus, plasmid pTPd3 was built using primers BamHI-YwkA and PstI-YwkA (see supplementary table) and introduced into *E. coli* M15 pREP4 to generate strain GTDp4, used for production of His<sub>6</sub>-YwkA. Storage buffer was the same as used for His<sub>6</sub>-YufM.

**Determination of kinetic parameters of YwkA.** YwkA malic enzyme activity was tested at 37°C by monitoring spectrophotometrically at 340 nm the NAD(P)H formation during the reductive decarboxylation of malate [malate+NAD(P) → pyruvate+NAD(P)H]. The reaction mixture was composed of 25 nmol YwkA in a Tris buffer containing divalent cations (50 mM Tris/HCl pH 8, 10 mM MgCl₂, 10 mM MnCl₂, 50 mM KCl, 10 mM β-mercaptoethanol). For determination of the *Kₘ* for malate, 5 mM of both cofactors were added to the reaction. For determination of *Kₘ* for each cofactor, the malate concentration was raised to 40 mM. For both cofactors, a molar absorption coefficient of 6-22 × 10<sup>4</sup> cm<sup>2</sup> mol⁻¹ was used for calculations. Demonstration of malic enzyme activity was assessed in a coupled reaction: at the end of the first reaction (end of NAD reduction), 40 units of strictly NAD-dependent lactate dehydrogenase from *Bacillus stearothermophilus* (Sigma) and 10 mM CaCl₂ were added to the reaction mixture. Then, oxidation of NADH, if produced by the first conversion of malate to pyruvate, was followed.

**DNA electrophoretic mobility shift assays.** The 277 bp DNA fragment of plasmid pPS61 containing the promoter region of *ywkA* was amplified by PCR with primers PS69 and PS70 (see supplementary table) and purified. Approximately 20 pmol of this fragment was end-labelled using T4 polynucleotide kinase (New England Biolabs) and 30 μCi (1-1 MBq) [γ-<sup>32</sup>P]ATP (Isoblu, ICN). After precipitation, 10,000 c.p.m. of radiolabelled probe (=0.05 pmol) was incubated for 5 min at room temperature in binding buffer (10 mM Tris/HCl pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5%, w/v, glycerol) supplemented with 1 μg herring sperm DNA. Varying amounts of His<sub>6</sub>-YufM were added for a total reaction volume of 20 μl. After incubating for 10 min at room temperature, samples were analysed by native 6% acrylamide gel electrophoresis, 1× Tris/glycine, run at 4°C and constant voltage (14 V cm⁻¹). After migration, gels were dried and the radiolabelled bands revealed by the Phospholmager system.

**DNase I footprinting.** Primers PS69 and PS70 (see supplementary table), 30 pmol each, were both end-labelled using T4 polynucleotide kinase (Biolabs) and 30 μCi (1-1 MBq) [γ-<sup>32</sup>P]ATP (Isoblu, ICN) and purified by precipitation. They were used to generate one-strand labelled PCR probes. The labelled DNA probes (60 000 c.p.m.; =0-25 pmol) were incubated for 15 min at room temperature with varying amounts of His<sub>6</sub>-YufM (the amount of protein was increased fivefold compared to gel mobility shift experiments due to partial loss of YufM binding activity after freezing). DNase I (Amersham Pharmacia Biotech) reactions were then performed as previously described (Doan & Aymerich, 2003). All samples were analysed on a 6% acrylamide sequencing gel, which was dried and revealed by autoradiography.
RESULTS

Transcriptome analysis reveals ywkA induction in the presence of malate

A transcriptome analysis originally designed to identify the *B. subtilis* genes whose expression varies depending on whether the central carbon flux is glycolytic or gluconeogenic was performed. Cells of strain 168CA grew at almost the same rate on either glucose or malate as the sole carbon source; their doubling times in S6 medium containing glucose and malate were 1.37 and 1.25 h, respectively. Thus, these growth conditions are suitable to detect by DNA microarray analysis specific changes in global gene expression during glycolytic growth versus gluconeogenic growth;

changes of expression of numerous genes such as ribosome protein genes that are affected by growth rate would not interfere.

Fig. 1 shows the expression ratio of genes involved in the glycolytic pathway and the TCA cycle between the gluconeogenic and the glycolytic growth conditions, which were inferred from our DNA microarray analysis (the complete microarray data are available on the KEGG web site: http://www.genome.ad.jp/kegg/expression). Expression of genes boxed in the figure was changed at least twofold between glycolysis and gluconeogenesis. *ywkA* transcription appeared to be induced more than sevenfold in the presence of malate as compared to glucose. The transcription signal of *ywkB*, the gene located just downstream and most probably constituting a bicistronic operon with *ywkA*, was very weak. Therefore, the upregulation of *ywkB* transcription found under gluconeogenic growth conditions (ratio 1.96) cannot be considered as significant. The gapB, pgkA and *pdhABC* genes were also upregulated under gluconeogenic growth conditions. By contrast, the gapA, pgk, tpi, eno and *yjmC* genes were upregulated during glycolytic growth. The upregulation of gapB (Fillinger et al., 2000) and *pckA* (Yoshida et al., 2001) during gluconeogenesis was reported previously. It has also been reported that transcription of the gapA, pgk, tpi and eno genes, as well as that of the *pgm* gene, both belonging to the same gapA-pgk-tpi-pgm-eno operon, is stimulated during glycolysis (Fillinger et al., 2000; Ludwig et al., 2001, 2002; Doan & Aymerich, 2003).

*ywkA* expression is induced only when malate is present in the medium

To confirm that *ywkA* transcription is induced in the presence of malate and to define more precisely the physiological conditions in which it is turned on, a reporter strain of the activity of the *ywkA* promoter region, PS1716, was constructed (Table 1). This strain harbours a *ywkA*::*lacZ* fusion inserted into the chromosome at the ectopic amyE locus. The β-galactosidase activity synthesised in strain PS716 grown in mineral medium supplemented with different carbon sources was measured (Table 2). The results

![Fig. 1. Expression change of the *B. subtilis* genes involved in glycolysis and gluconeogenesis. Genes involved in the glycolytic pathway and the TCA cycle are followed by their expression ratios (malate/glucose) obtained by the DNA microarray analysis. The expression ratio for each gene was taken as the mean of two ratios obtained from the different hybridization profiles. Genes whose expression was changed at least twofold between glycolytic and gluconeogenic growths, are boxed (glycolytic genes) or boxed and stippled (gluconeogenic genes). The underlined gapA, pgk, tpi, pgm and eno genes, on the one hand, and pdhABC genes, on the other, are organized in two operons. The complete microarray data are available on the KEGG website (http://www.genome.ad.jp/kegg/expression).](http://mic.sgmjournals.org)
indicated that ywkA promoter activity was very weak in the presence of succinate plus glutamate, or fumarate, and of glucose or glycerol, as sole gluconeogenic and glycolytic carbon source, respectively. Similarly, very weak basal β-galactosidase activity was detected in the presence of other gluconeogenic carbon sources, pyruvate or aspartate (data not shown). By contrast, the expression of the reporter construct was very strong in the presence of malate (and rather constant throughout the exponential growth phase). Moreover, addition of glucose to malate minimal medium did not affect ywkA expression. These results showed that ywkA transcription is specifically induced in the presence of malate in the culture medium and that it is not subject to carbon catabolite repression. Furthermore, because no expression was detected in the presence of fumarate, a C4-dicarboxylic acid that enters the TCA cycle and is then converted in one step into intracellular malate, it could be assumed that only extracellular malate induces ywkA expression.

**ywkA does not contribute to the utilization of malate for growth**

As ywkA is specifically expressed when malate is present in the culture medium, we wanted to find out whether this gene is involved in the utilization of malate as a carbon source for growth. A ywkA mutant strain, GTD102, was constructed and was tested for growth in minimal medium containing either malate, or succinate plus glutamate (entering the TCA cycle and subsequently providing malate), or a glycolytic carbon source, glucose. This growth test showed that strain GTD102 was able to use either malate (Fig. 2), or succinate plus glutamate or glucose (data not shown) as efficiently as the wild-type strain.

A ywkA inactivation might be functionally complemented by one or several other putative malic enzyme encoding genes. Therefore, we also constructed different strains harbouring different combinations of mutations of the ywkA, malS and ytsf genes: single mutant strains BFA91 (ytsf) and GTD110 (malS), double mutant strains GTD111 (ywkA, malS), GTD121 (ywkA, ytsf) and GTD122 (ytsf, malS), and a triple mutant strain GTD123 (ywkA, malS, ytsf). All these strains were able to grow in glucose minimal medium as efficiently as the wild-type strain (data not shown). But when growing in the presence of malate as sole carbon source (Fig. 2), all the strains mutated for ytsf, BFA91, GTD121, GTD122 (data not shown) and GTD123, exhibited the same lower growth rate than the wild-type strain. By contrast, all the other mutants, GTD102, GTD110 and GTD111 (data not shown) grew as well as the wild-type strain. The ywkA mutation is supposed to have a polar effect on ywkB, and the absence of phenotype could be due to the absence of expression of both ywkA and ywkB. Because the ywkA disruption was constructed using a pMUTIN derivative (Moriya et al., 1998; Vagner et al., 1998), the possible polar effect on ywkB can be suppressed by addition of IPTG. However, the growth pattern of the strains harbouring a ywkA mutation was identical in the presence and in the absence of IPTG (data not shown). These experiments confirmed that ywkA and malS are not necessary for growth under either of the conditions tested. By contrast, ytsf appeared to play a major role in the utilization of malate for growth.

**Ywka exhibits a malic enzyme activity and preferentially utilizes NAD**

Does ywkA encode a functional malic enzyme? To answer this question, a characterization of the enzymic properties of the Ywka protein was performed. First, a vector designed to overexpress a Ywka tagged with His6 was constructed (pTDp3) and introduced into *E. coli* M15 pREP4. Overexpression was induced by cultivating the resulting strain in the presence of IPTG, and the His6-Ywka protein was purified to apparent homogeneity. Then, the capacity of this protein to reduce NAD or NADP in the presence of malate was assayed. The results showed that Ywka has a strong activity of reduction of either NAD+ or NADP+ that depends on the presence of malate. To demonstrate that this reducing activity is associated with the conversion of malate into pyruvate, i.e. that Ywka exhibits a malic enzyme activity and not a malate dehydrogenase or a malolactic enzyme activity, the assay of the activity of an enzyme, lactate dehydrogenase, using specifically pyruvate as substrate, was coupled to the assay of Ywka. Indeed, addition of lactate dehydrogenase after the end of the first reaction (no further increase of 

![Fig. 2.](attachment:compare_growth strains.png)

*Fig. 2.** Comparative growth of strains 168CA (○), GTD102 (ywkA, □), BFA91 (ytsf, △), GTD110 (malS, △), GTD121 (ytsf ywkA, ◇) and GTD123 (ytsf ywkA malS, ○) in malate minimal medium (MM malate).*
determined to be 6·6 mM. $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values were 1·1 mM, 112 s$^{-1}$, and 102 s$^{-1}$ mM$^{-1}$, respectively, for NAD, and 9·0 mM, 88 s$^{-1}$, and 10 s$^{-1}$ mM$^{-1}$, respectively, for NADP. Thus, we concluded that YwkA is a malic enzyme with a dual specificity for NAD and NADP as a cofactor but with a strong preference for NAD [10-fold higher catalytic efficiency ($k_{cat}/K_m$) with NAD than with NADP].

**Transcriptional characterization of ywkA**

RNA samples prepared from cells of strains 168CA, 1A250 and 1A147 (ccpA1) grown in malate or glucose minimal medium were subjected to Northern analysis using specific probes for ywkA, ywkB, and gapB, a key gluconeogenic gene. As shown in Fig. 4, a specific transcript (2·8 kb) was detected with the ywkA probe only when cells were grown on malate (lanes 2, 4 and 6). This transcript is able to cover the ywkA gene as well as the downstream gene, ywkB. When the ywkB probe was used, this 2·8 kb transcript was only slightly visible due to the presence of the faint broad bands from rRNAs (3·0 and 1·5 kb), only when cells of the three strains were grown on malate (lanes 2, 4 and 6). These results strongly suggested that the ywkAB genes are cotranscribed as a single transcription unit. As a control, a transcript (1·7 kb) of gapB was observed, as expected, only when cells were grown on malate (Fig. 4, gapB lanes 2, 4 and 6) (Fillinger et al., 2000). Furthermore, this analysis also confirmed that repression of ywkA-ywkB transcription as well as gapB transcription (Fillinger et al., 2000; Yoshida et al., 2001) is independent of CcpA (compare lanes 4 and 6).

Primer extension analysis mapped a 5′-end of the ywkAB transcript only with RNA prepared from cells of strain 168CA grown on malate, not from those grown on glucose (Fig. 5, lane 1), and allowed us to find a corresponding promoter sequence consisting of −10 (TACAAT) and −35 (TTGAAT) regions separated by a 17 bp spacer, which is probably recognized by $\sigma^A$ RNA polymerase. No other clear 5′-end of the ywkAB transcript was detected in the region (nucleotides about +120 to −140) covering the translation initiation nucleotide of ywkA (+80) by our primer extension experiments, including ones using another more downstream primer (data not shown).

In conclusion, the ywkAB genes are cotranscribed to produce a 2·8 kb transcript from the transcription initiation site (Fig. 5); this transcription is induced during growth on malate and this regulation is independent of CcpA.

**Genetic screening for regulator(s) involved in the control of ywkA transcription**

The ywkAB genes are not physically linked to any gene encoding a putative transcriptional regulator. So, we decided to search for a regulator of ywkAB transcription by a genetic screening. A mini-Tn10 transposant library was obtained.
from *B. subtilis* strain PS1716, which contains an ectopic ywkA′ :: lacZ reporter fusion. This library was then screened on plates for deregulation of ywkA expression (see Methods). Several clones from independent pools of transposants were isolated in which ywkA was no longer induced in the presence of malate. The gene targeted by the transposon in 18 of these clones was identified. YufL was the gene most frequently found to be interrupted (eight occurrences). Back-crossing experiments from these eight transposants indicated that the loss of ywkA inducibility was associated with the insertion of the transposon. As predicted on the basis of its nucleotide sequence, yufL would encode the membrane-bound sensor-kinase of a TCS, associated with the transcriptional response-regulator encoded by the putatively co-transcribed gene yufM. However, no clone harbouring a transposon inserted into the yufM ORF was picked up in this genetic screening.

**The TCS YufL/YufM is required for ywkA induction by malate**

To confirm the role of the YufL/YufM TCS in the regulation of ywkA transcription, we introduced a yufM disruption into strain PS1716 and analysed the expression of the ywkA′ :: lacZ reporter fusion in the resulting strain (GTD132). Strain GTD134, a PS1716-derived strain in which the yufL gene is disrupted by a plasmidic cassette that places the downstream gene yufM under the control of a Pspac promoter, and the parental strain PS1716 were also tested in parallel. The β-galactosidase activity synthesized by these strains grown in the presence of glucose or malate as sole carbon source was measured (Table 3). The results showed that ywkA expression was no longer induced by malate in a yufL or yufM mutant strain. In the yufL mutant strain (GTD134), expression of ywkA was restored after the artificial overexpression of yufM by addition of IPTG, GAT C 12

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity* [U (mg protein)-1]</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Malate</td>
<td></td>
</tr>
<tr>
<td>PS1716 [amyE′ :: PywkA :: lacZ]</td>
<td>8</td>
<td>655</td>
<td></td>
</tr>
<tr>
<td>GTD134 [amyE′ :: PywkA :: lacZ, yufL′ :: pEC23(kan)]</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>GTD134 [amyE′ :: PywkA :: lacZ, yufL′ :: pEC23(kan)]</td>
<td>250 (+ IPTG)</td>
<td>368 (+ IPTG)</td>
<td></td>
</tr>
<tr>
<td>GTD132 [amyE′ :: PywkA :: lacZ, yufM′ :: pEC23(kan)]</td>
<td>5</td>
<td>7</td>
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*Activity was assayed at least three times from independent clones; representative results from one series are shown. Strains were cultivated in glucose or malate CQTHC media (+ IPTG when indicated).
independently of the carbon source, glucose or malate, present in the medium. These findings thus demonstrated that the YufL/YufM TCS is required for ywkA induction in response to the presence of malate.

The growth rate and the biomass yield of the yufM and of the yufL mutant strains in the malate medium was significantly lower than that of the parental strain (doubling time 1·6 h versus 1·2 h; final OD_{600} 0·7 versus 1·5, respectively). This observation suggests that the YufL/YufM TCS positively controls the expression of other genes that, in contrast to ywkA, are involved in the utilization of malate as carbon source for growth.

**YufM is able to bind specifically to the regulatory region of ywkA**

A His-tagged YufM protein was overexpressed in *E. coli* and purified to apparent homogeneity (a figure showing the purification is available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). A gel mobility shift assay was then performed to test the capacity of YufM to bind to the *ywkA* regulatory region. The assay, which was carried out in the presence of a large excess of unspecific DNA competitor, showed that YufM can bind directly and specifically to the regulatory region of *ywkA* in a concentration-dependent manner (Fig. 6). The shift of the probe was complete only when the protein/DNA concentration ratio exceeded 150; this suggests that, as demonstrated for other TCS response regulators (Perego & Hoch, 2002), YufM requires to be phosphorylated to be fully active in DNA binding.

**YufM protects a 50 bp DNA sequence upstream of the *ywkA* promoter sequence**

To define more precisely the YufM binding region, DNase I footprinting experiments were performed. The 277 bp fragment present upstream of the *ywkA* coding sequence was labelled on one strand or the other, incubated with an increasing amount of YufM, and then hydrolysed by DNase I. As revealed after electrophoresis (Fig. 7a), YufM protected a 50 bp sequence shifted three bases upstream of the promoter. Thus we propose to rename this TCS MalK/MalR for malate sensor kinase/malate regulator component. On the other hand, if our enzymic study clearly demonstrated that YwkA is a malic enzyme, our phenotypic analysis showed that *ywkA* is not necessary for utilization of malate as sole carbon source for growth.

Interestingly, our transcriptome analysis indicated that either *malS* or *ytsJ*, two paralogues of *ywkA*, were significantly and similarly transcribed in the presence of glucose or malate, suggesting that their expression is not regulated by malate nor by the glycolytic or gluconeogenic carbon fluxes.

![Fig. 6. Specific binding of His{sub}_6-YufM to the promoter region of the *ywkA* operon. The 277 bp DNA fragment inserted into plasmid pPS61 (approx. 0·05 pmol) was labelled and then incubated with no or increasing amounts of His{sub}_6-YufM in the presence of a large excess of unspecific DNA.](http://mic.sgmjournals.org)
orientation. By contrast, ywkA transcription was induced only in the presence of malate in the culture medium. Further addition of glucose or another glycolytic carbon source to the medium did not affect ywkA expression. Thus, ywkA is not subject to carbon catabolite repression. YwkA was not expressed during growth on fumarate or succinate plus glutamate, although these compounds enter the TCA cycle upstream of malate and thus very probably lead to the elevation of the intracellular malate concentration. It is thus very likely that extracellular malate is the signal for ywkA induction. Of course this does not exclude the possibility that the permeation of malate would be necessary for efficient transduction of the signal and thus full induction of ywkA transcription. The effects of the inactivation of the different putative malate transporters on the inducibility of ywkA need to be studied to test this possibility.

To try to get some clues concerning the biological role of ywkA, the regulatory mechanism of its expression has been investigated. Our analysis demonstrated that the YufL/YufM TCS is the positive regulatory system that mediates the induction of ywkA expression in response to the presence of malate in the culture medium, YufM being a direct transcriptional activator of ywkA transcription. This conclusion is in agreement with the results of a global transcriptome analysis performed by Kobayashi et al. (2001)
indicating that ywkA expression is ninefold upregulated in a yufL mutant strain overexpressing YufM, compared to a wild-type strain. The YufL/YufM TCS belongs to the CitA/CitB family of TCSs. The members of this family that have already been characterized are CitA/CitB from E. coli (Kaspar & Bott, 2002) and Klebsiella pneumoniae (Bott et al., 1995; Kaspar et al., 1999), CitS/CitT from B. subtilis (Yamamoto et al., 2000) and Streptomyces coelicolor, YdbF/YdbG from B. subtilis (Asai et al., 2000) and DcuS/DcuR from E. coli (Zientz et al., 1998; Golby et al., 1999). All these TCSs are involved in the control of genes required for TCA cycle intermediates import and utilization. Sequence analyses strongly suggest that YufL is a transmembrane protein containing an extracellular sensor domain and a cytoplasmic kinase domain, as shown for the highly similar E. coli DcuS protein (Golby et al., 1999). Thus, YufL would be able to modulate YufM DNA-binding activity by phosphorylation/dephosphorylation depending on the presence of extracellular malate. The transcriptome analysis performed by Kobayashi et al. (2001) has revealed 97 other differentially expressed genes in the yufL mutant strain overexpressing yufM compared to the wild-type strain. Because this list included several competence genes, and especially comK and most of the members of the ComK regulon (Berka et al., 2002; Ogura et al., 2002), the authors suggested that the YufL/YufM TCS would be involved in competence development. However, we found no drastic transformability defect, if any, of yufL, yufM or ywkA mutant strains (T. Doan & S. Aymerich, unpublished data). The study of the genome-wide expression changes dependent on ComK, recently reported by Berka et al. (2002), led these authors to conclude that the ComK regulon defines a growth-arrested state, distinct from sporulation, of which competence for genetic transformation is but one notable feature. They suggest that this is a unique adaptation to stress and that it be termed the ‘K-state’. Thus the YufL/YufM TCS could participate in the signalling of the ‘K-state’.

Interestingly, simultaneously with our work, the expression of maeN (yufR), a gene located 5-3 kb downstream of yufL-yufM on the chromosome, has been found to be activated by the YufL/YufM TCS in the presence of malate (Tanaka et al., 2003). Wei et al. (2000) had previously reported that MaeN exhibits a malate–Na + symporter activity. Ogasawara and colleagues indeed found that MaeN, and thus YufL/YufM, are essential for utilization of malate as sole carbon source for growth (Tanaka et al., 2003). This is in agreement with the growth defect on malate associated with the yufL and the yufM mutations that we observed during the test of their effects on ywkA expression; however, the defect was only partial in our experiments because the medium used contained not only malate but also glutamine and casein hydrolysate as carbon sources (Table 3). The coregulation of ywkA and maeN is of course physiologically relevant since MaeN could provide the YwkA malic enzyme with cytoplasmic malate. Furthermore, Krulwich and coworkers (Ito et al., 2000) have shown that, in some particular conditions, maeN expression was coupled to the expression of the downstream mrp gene cluster involved in alkali resistance and pH homeostasis. These authors suggested a possible requirement of coordinated Na +–malate symport and Na + re-extrusion in exchange for H +. The protonmotive force could be generated by secondary NADH dehydrogenases putatively encoded by some mrp genes. Therefore, YwkA could be involved in the regeneration of NADH during this process.

Ogasawara and colleagues have also found that the expression of yflS, putatively encoding a 2-oxoglutarate/malate translocator, was under the control of the YufL/YufM TCS (Tanaka et al., 2003). Indeed, maeN and yflS have been detected as being up-regulated (ratios Mal/Glc 4-2 and 2-7, respectively) during growth on malate in our transcriptomic analysis. By contrast, they were not clearly identified (ratios 1-7 and 1-5) in the transcriptomic analysis of Kobayashi et al. (2001), which compared a yufL mutant strain overexpressing YufM with the wild-type strain. We have compared the sequence of the region protected by YufM upstream of the ywkA, the maeN and the yflS promoters and found no significant similarities. The most conspicuous feature of these three regions is their very high A + T content (88, 83 and 82 mol%, upstream of ywkA, maeN and yflS, respectively). On the other hand, no other occurrence of one or the other direct repeat included in the region protected by YufM upstream of ywkA (Fig. 6) can be detected in the complete B. subtilis genome. Thus one should conclude that the YufM DNA-binding activator does not recognize a unique primary sequence, a proper similarity to that of several DNA-binding components of other well-characterized TCSs (Perego & Hoch, 2002).

YwkA does have malic enzyme activity but it does not contribute to the very efficient utilization of malate as sole carbon source for growth by B. subtilis. This is in agreement with the fact that ywkA is not subject to carbon catabolite repression. Because neither a malS nor a double ywkA malS mutant strain showed any growth defect, it can also be concluded that, like ywkA, malS is not necessary for growth on malate. By contrast, the ytsJ gene is required for efficient growth on malate, as well as on other TCA cycle intermediates. YtsJ is expressed constitutively during the exponential phase of growth and encodes a NADP-dependent malic enzyme (T. Doan, G. Lerondel & S. Aymerich, unpublished data). YtsJ could thus be required for its capacity to catalyse an anaplerotic reaction that allows the TCA cycle to function as a real cycle when the only carbon source is one intermediate of this cycle, or for its capacity to regenerate NADPH necessary for numerous anabolic reactions, or for both of these activities. We would propose that ywkA is involved in a more specific biological process necessary only or principally when malate is present in the extracellular environment. The study of the ywkB gene, of which no clear orthologue can be detected in the genome of other bacilli, and the identification of the activity of more genes belonging to the yufL/YufM TCS regulon, should help to develop this hypothesis.
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