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 (\(k_{cat}/K_m\) 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 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 isoforms. In *Escherichia coli*, both NAD- and NADP-dependent malic enzymes, named SfcA 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 SfcA is involved in gluconeogenesis by providing pyruvate, then converted into...
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 nitrogen fixation as a result of its role for growth in the

The examination of the sequence of the B. subtilis chromosome indicates that ywka is probably cotranscribed with ywbB, which encodes a protein of unknown function. malS is linked to ytsP, an unknown gene, and ytsI 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 (TCSSs) 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 TCSSs 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 TCSSs 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 ytsI. The regulation of the expression of the ywka gene has therefore been further investigated, and the uncharacterized YufL/YufM TCSS 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 TGI [K12 Δ(lac–pro) supE thi hsdR−5F′ traD36 proA2 B’− lacIq lacZAM15] used as a general cloning host and E. coli strain M15 pREP4 (Qigagen) 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 mg l⁻¹;

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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 (chlor-
ampenicol), 0-4 mg l⁻¹ (erythromycin), 100 mg l⁻¹ (spectomy-
cin), 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
with the QIAprep spin miniprep kit (Qiagen). DNA fragments were purified from agarose gels using
with the Qiagen Gel Extraction Kit.

Oligonucleotides. The oligonucleotides used in this study are
listed in a table available as supplementary data with the online
version of this paper (http://mic.sgm.journals.org).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
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<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>ytsf'::pMUTIN2</td>
<td>BSFA† collection</td>
</tr>
<tr>
<td>GTD102</td>
<td>ywka'::kan</td>
<td>pEC23→YWKAd</td>
</tr>
<tr>
<td>GTD110</td>
<td>ΔmalS::spc</td>
<td>This study</td>
</tr>
<tr>
<td>GTD111</td>
<td>ywka'::kan ΔmalS::spc</td>
<td>GTD102 DNA × GTD110</td>
</tr>
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<td>ytsf'::pMUTIN2 ywka'::kan</td>
<td>BFA91 DNA × GTD102</td>
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<td>BFA91 DNA × GTD110</td>
</tr>
<tr>
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<td>1A250</td>
<td>alslR1 ivlA1</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>1A147</td>
<td>ccppA1 alslR1 ivlA1</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
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<td>This study</td>
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<td>yuaL'::pMUTIN2</td>
<td>BSFA collection</td>
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<td>pEC23→BFA1431</td>
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<td>yufM'::pMUTIN2</td>
<td>This study</td>
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<td>GTD131</td>
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<td>GTD132</td>
<td>[pMUTIN2 Δ(lacZ–ery)::pEC23( kan)] amyE::PywkA'::lacZ–cat</td>
<td>GTD131 DNA × pPS1716</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.

**Construction of strains.** A fusion of a ywka promoter fragment
to a promoter-less lacZ gene was constructed using the vector
pDG1661 (Guérout-Fleury et al., 1996). The 277 bp promoter frag-
ment to be tested was obtained by PCR using primers PS69 and
PS70 (see supplementary table); this fragment was inserted between
the EcoRI and BamHI sites of pDG1661 to generate plasmid pPS61,
then used to insert the reporter construct into the B. subtilis amyE
locus to generate strain PS1716 (Table 1).

In vivo replacement by homologous recombination of a kanamycin-
resistance cassette for the lacZ ORF and erythromycin resistance gene
of pMUTIN was obtained using pEC23, a plasmid specifically designed
for this use, constructed by P. Stragier, Institut de Biologie Physico-
Chimique, 75005 Paris, France.

Strain GTD110 was constructed by replacing the malS ORF by the
spectinomycin-resistance cassette contained between the BamHI and
XbaI sites of plasmid pCS33 (Steinmetz & Richter, 1994): two PCR
fragments, one corresponding to the 500 bp upstream of malS ORF
and one to the 500 bp downstream of the malS ORF, were generated
with primer pairs EagI-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 pH101 (Ferrari et al.,
1982) to generate plasmid pTD101, 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
 glucose or 37-5 mM malate. Total RNAs were isolated from cells
esentially as previously described (Yoshida et al., 2001). For Northern
blot analysis, RNA samples (2 μg each) were electrophoresed in
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 Bac BEST labelling kit (Takara Shuzo) and [α-32P]dCTP (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 Cy5 and Cy3 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 Cy5 and Cy3 intensities, we calculated the expression ratios.

To get reliable results, 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 [α-32P]dCTP (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 PS1716 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 casein hydrolysate (0.05%) or LB medium, plus X-Gal supplemented with glucose (0-5%) or maltose (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 o-nitrophenol min⁻¹ at 20°C.

**Expression and purification of His₆-YuFM.** His₆-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 PsiI-YuFM (see supplementary table) in order to produce a fusion protein with a hexahistidine tag at the N-terminus. The resulting plasmid, pTDp3, was transformed into E. coli M15 pREI4401 to generate the strain used for production, GTDp3. All the subsequent procedures were performed as described in the QiAexpress manual. His₆-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 His₆-YwkA.** His₆-YwkA was overexpressed and purified as described for His₆-YuFM. Thus, plasmid pTDP3 was built using primers BamHI-YwkA and Sphl-YwkA (see supplementary table) and introduced into E. coli M15 pREP4 to generate strain GTDp4, used for production of His₆-YwkA. Storage buffer was the same as used for His₆-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 mM 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 and Vₘₐₓ for each cofactor, the malate concentration was raised to 40 mM. For both cofactors, a molar absorption coefficient of 6.22×10⁶ cm⁻¹ 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-32P]ATP (Isoblue, ICN). After precipitation, 10000 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 His₆-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 Phosphoimager system.

**DNase I footprinting.** Primers PS69 and PS70 (see supplementary table), 30 pmol each, were both end-labelled using T4 polynucleotide kinase (Biotools) and 30 µCi (1-1 MBq) [γ-32P]ATP (Isoblue, ICN) and purified by precipitation. They were used to generate one-strand labelled PCR probes. The labelled DNA probes (60000 c.p.m.; ~0-25 pmol) were incubated for 15 min at room temperature with varying amounts of His₆-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 website: 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 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 synthesized in strain PS1716 grown in minimal medium supplemented with different carbon sources was measured (Table 2). The results

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**Table 2.** Expression of a ywkA::lacZ reporter fusion in minimal medium supplemented with various carbon sources

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>β-Galactosidase activity* [U (mg protein)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>9</td>
</tr>
<tr>
<td>Fumarate</td>
<td>11</td>
</tr>
<tr>
<td>Succinate</td>
<td>17</td>
</tr>
<tr>
<td>Malate + glucose</td>
<td>764</td>
</tr>
<tr>
<td>Malate + glucose</td>
<td>764</td>
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</tbody>
</table>

*Activity was assayed at least three times from independent clones; representative results from one series are shown. Strains were cultivated in CQTHC medium supplemented with the carbon sources indicated.
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 other putative malic enzyme encoding genes. Therefore, we also constructed different strains harbouring different combinations of mutations of the ywkA, malS and ytsJ genes: single mutant strains BFA91 (ytsJ) and GTD110 (malS), double mutant strains GTD111 (ywkA, malS), GTD121 (ywkA, ytsJ) and GTD122 (ytsJ, malS), and a triple mutant strain GTD123 (ywkA, malS, ytsJ). 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 ytsJ, 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, ytsJ 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 that depends on the presence of malate was assayed. The results showed that YwkA has a strong activity of reduction of either NAD+ or NADP+ that is coupled to the assay of YwkA. Indeed, addition of lactate dehydrogenase after the end of the first reaction (no further increase of A430) led to rapid reoxidation of NADH (Fig. 3). This could result only from oxidative conversion of pyruvate into lactate and thus demonstrated that pyruvate is the product of the first reaction catalysed by YwkA. In a second series of experiments, we determined the kinetic parameters of YwkA activity. The $K_m$ value for malate was
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<sup>9</sup>::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<sup>9</sup>::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.
malic enzyme activated by YuflM TCS

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 *ywkA* promoter. 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 *ywkA* promoter.

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 flux

**DISCUSSION**

In this study, we have investigated the physiological function and the transcriptional regulation of *ywkA*, one of the four paralogous genes of *B. subtilis* that could be predicted to encode malic enzymes. On the basis of the results of a global transcriptomic analysis using DNA microarrays, we have further studied the expression pattern of the *ywkA* gene through *lacZ* reporter fusion, Northern and primer extension analyses. We have been able to demonstrate that *ywkA* expression is specifically induced by malate. A genetic study then identified the uncharacterized YuflL/YufM TCS as the regulatory system necessary for the activation of *ywkA* transcription in response to the presence of malate in the culture medium. In *in vitro* experiments demonstrated that YufM is able to bind directly to a region located just upstream of the *ywkA* 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.

The enzymic characterization reported here demonstrated that YwkA exhibits a malic enzyme activity and excludes the possibility that it is a malolactic enzyme or a malate dehydrogenase. Furthermore, it showed that YwkA has a 10-fold higher catalytic efficiency with NAD as a cofactor than with NADP. We therefore propose to class YwkA in the group of NAD-dependent malic enzymes, EC 1.1.1.38, and to rename its structural gene *maeA* for malic enzyme A.

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 flux

![Fig. 6. Specific binding of His<sub>6</sub>-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</sub>-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 a 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 property similar to that of several DNA-binding components of other well-characterized TCSs (Pereg & 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 *maIS* nor a double *ywkA maIS* mutant strain showed any growth defect, it can also be concluded that, like *ywkA*, *maIS* 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 an 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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