Analysis of the effect exerted by extracellular pH on the maltose regulon in *Escherichia coli* K-12

Sylvie Alonzo, Martine Heyde, Patrick Laloi and Raymond Portalier

Author for correspondence: Patrick Laloi. Tel: +33 472 431 621. Fax: +33 472 431 971.
e-mail: plaloi@biom.serv.univ-lyon1.fr

Laboratoire de
Microbiologie et
Génétique Moléculaire,
Centre de Génétique
Moléculaire et Cellulaire,
UMR CNRS 5534,
Université Claude Bernard,
69622 Villeurbanne Cedex,
France

The *Escherichia coli* maltose regulon consists of five operons under the control of the MalT transcriptional activator. *lac* operon fusions were constructed *in vitro* with the MalT-dependent promoter and with the *malT* promoter itself. β -Galactosidase activity displayed by these fusions during growth at different external pH (pH_o) revealed that growth at a pH_o higher than 6 stimulates the transcription of *malT*- and MalT-controlled genes in the absence or presence of maltose. Using a *malTp1 malTp10* promoter that is cAMP-CRP (cAMP receptor protein)-independent, it was demonstrated that CRP is essential for *malT* pH_o regulation and that the pH_o -dependent activity of *malKp* is a direct consequence of *malT* regulation. The pH_o regulation displayed by a deleted but still functional *malT* promoter fused to *lacZ* demonstrates that this minimal promoter contains all the regulatory regions for establishing pH_o regulation. In the absence of Mlc, a repressor of *malT* expression, the pH_o regulation of *malT* was still effective. It is proposed that binding of cAMP-CRP at *malTp* may be affected by *malTp* topology induced by pH_o or that a pH_o -dependent effector may act in concert with the cAMP-CRP complex.

Keywords: *Escherichia coli*, transcription, cAMP-CRP, maltose regulon, pH

INTRODUCTION

To contribute to the understanding of the complex molecular interplay between the different regulatory mechanisms and environmental signals that influence gene expression, we have studied the influence of external pH (pH_o) on the expression of the *Escherichia coli* K-12 maltose regulon. *E. coli* has a constitutive homeostatic mechanism which allows cells to maintain their internal pH between 7.4 and 7.8 over a pH_o range of 5.0–8.5 (Zilberstein *et al.*, 1984). Therefore, within this pH_o range, regulation of gene expression in response to pH_o must involve mechanisms capable of sensing and transducing the pH_o stimulus. In *E. coli*, the level of expression of many genes is influenced by the pH_o (for a review, see Olson, 1993). We have described *in vivo* gene fusions whose expression is increased during growth at high pH_o compared to low pH_o (Heyde *et al.*, 1991). The *malB* region, which belongs to the maltose regulon, was identified among the different pH_o -modulated loci.

The maltose regulon of *E. coli* consists of numerous genes whose products are involved in the transport and catabolism of maltose and maltodextrins (Schwartz, 1987; Boos *et al.*, 1996). These genes belong to five transcriptional units under the control of a transcriptional activator, the MalT protein (Richet & Raibaud, 1989). Three MalT-dependent operons (*malPQ*, *malS* and *malZ*) are independent of the cAMP receptor protein (CRP), whereas the other two, *malK-lamB-malM* and *malEFG* require the cAMP-CRP complex in addition to MalT (Chapon, 1982). MalT appears monomeric in solution and, in the absence of inducer, is in an inactive form (MalT_i). The binding of ATP (K_D 0.4 μM ; Richet & Raibaud, 1989) and maltotriose (K_D about 20 μM ; Raibaud & Richet, 1987) to MalT_i stabilizes active MalT (MalT_a) for transcriptional activation. *malT* expression is not autoregulated, but is subject to catabolite repression and requires the presence of the cAMP-CRP complex (Chapon, 1982; Chapon & Kolb, 1983). It is also controlled by a repressor called Mlc, a putative new global regulator for sugar metabolizing systems. In the absence of Mlc, *malT* expression is increased by a factor of 2–3 when *E. coli* is grown in glycerol (Decker *et al.*, 1998). Mlc binds to *malT* DNA

Abbreviations: CRP, cAMP receptor protein; MM, minimal medium; pH_o , external pH.

Table 1. Bacterial strains and plasmids

Genetic nomenclature is from Bachmann (1990). Transductants were selected for the first phenotype indicated and analysed for the second one. Tet^R, Kan^R, Cam^R, Amp^R indicate resistance to tetracycline, kanamycin, chloramphenicol or ampicillin, respectively. Mtl⁺ indicates growth with mannitol as carbon source.

Strain/plasmid	Relevant genotype	Source or reference
Strains cited in the figures or in the text		
GPH1767	MC4100(pGPH1767)	This work
GPH1768	MC4100(pGPH1768)	This work
GPH8561	pop3325(pGPH1768)	This work
GPH8805	MC4100 (<i>ompA::lacZ</i>)hyb	Kan ^R with P1 on JC4948
GPH8818	pop4125(pGPH1768)	This work
GPH8863	GPH8862(pGPH1768)	This work
GPH8879	MC4100(pGPH8879)	This work
GPH8881	MC4100(pGPH8881)	This work
GPH8888	GPH1768 <i>malQ::Tn10</i>	Tet ^R with P1 on pop4064
GPH8892	GPH8549(pGPH8881)	This work
GPH9274	MC4100(pGPH9261)	This work
GPH9275	MC4100(pGPH9260)	This work
GPH9463	MC4100(pGPH9463)	This work
GPH9949	GPH8881 <i>mlc::Tn10</i> Kan	Kan ^R with P1 on KD612
SF1707	MC4100 Φ (<i>mals'-lacZ</i> ⁺)1 Mud1(Amp <i>lac</i>)	Freundlieb & Boos (1986)
Strains used as starting material or as an intermediate during the construction of the above strains		
BG525	Δ <i>cya06</i> Δ <i>crp45</i> <i>thi</i>	Institut Pasteur, Paris
BS524	Δ <i>cya06</i>	J. Beckwith, Harvard Medical School
BS680	<i>thi</i> Δ <i>crp45</i> <i>aroB</i>	Institut Pasteur, Paris
CA8404	<i>crp*</i> <i>rpsL</i>	J. Beckwith, Harvard Medical School
CGSC6419	F ⁻ Δ (<i>lacZY</i>)286 <i>zie296::Tn10</i> <i>xyl-7</i> <i>mtl-1</i> <i>argH1</i> λ^-	B. Bachmann, Yale University
GPH6794	BS680 <i>zhe::Tn10</i> <i>aroB</i>	Tet ^R with P1 on K311/1
GPH6977	BS524 <i>zie296::Tn10</i>	Tet ^R with P1 on CGSC6419
GPH6999	CA8404 <i>zhe::Tn10</i>	Tet ^R with P1 on GPH6794
GPH7268	BG525 <i>crp*</i> <i>zhe::Tn10</i>	Tet ^R with P1 on GPH6999
GPH8513	MC4100 Δ <i>cya</i> <i>zie296::Tn10</i>	Tet ^R with P1 on GPH6977
GPH8549	GPH8513 <i>crp*</i>	Mtl ⁺ with P1 on GPH7268
GPH8820	TE2680 <i>malK::Kan^R</i>	This work
GPH8862	MC4100 <i>malK::Kan^R</i>	Kan ^R with P1 on GPH8820
JC4948	Hfr P4X <i>thi</i> <i>metB1</i> <i>relA1</i> <i>spoT1</i> Δ (<i>lac</i>)U169 (<i>ompA::lacZ</i>)hyb(pNK629)	This laboratory
K311/1	K311 <i>aroB</i> <i>zhe::Tn10</i>	V. Braun, University of Tübingen
KD612	MC4100 Φ (<i>malT'-lacZ</i>)1110 (λ placMu50) <i>mlc::Tn10</i> Kan	Decker <i>et al.</i> (1998)
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)205 λ^- <i>flhD5301</i> <i>fruA25</i> <i>relA1</i> <i>rpsL150</i> <i>rbsR22</i> <i>deoC1</i>	M. Casadaban, University of Chicago
pop3325	MC4100 <i>malT^c1</i>	Institut Pasteur, Paris
pop4064	MC4100 <i>malQ::Tn10</i>	E. Richet, Institut Pasteur, Paris
pop4125	MC4100 <i>malTp1</i> <i>malTp10</i> /F ⁺	E. Richet, Institut Pasteur, Paris
pop4127	MC4100 <i>malTp1</i> <i>malTp10</i> <i>zhd::Tn10</i> Δ <i>crpT8</i> /F ⁺	E. Richet, Institut Pasteur, Paris
TE2680	F ⁻ λ^- IN(<i>rrnD-rrnE</i>)1 Δ (<i>lac</i>) χ 74 <i>rpsL</i> <i>galK2</i> <i>recD1903::Tn10d</i> -Tet <i>trpDC700::putPA1303::[Kan^R-Cam^R-lac]</i>	Elliott (1992)
Plasmid		
pBR328	Amp ^R Tet ^R Cam ^R	Valentin-Hansen <i>et al.</i> (1986)
pJEL249	Amp ^R (30 μ g ml ⁻¹). Temperature-sensitive copy number. <i>lacZYA</i> gene fusion vector	

Table 1. (cont.)

Strain/plasmid	Relevant genotype	Source or reference
pJEL250	Amp ^r (30 µg ml ⁻¹). Temperature-sensitive copy number. <i>lacZYA</i> operon fusion vector	Valentin-Hansen <i>et al.</i> (1986)
pGPH1767	Φ(<i>malE'</i> - <i>lacZ</i> ⁺)1 in pJEL250	This work
pGPH1768	Φ(<i>malK'</i> - <i>lacZ</i> ⁺)1 in pJEL250	This work
pGPH8579	See Methods	This work
pGPH8579K	See Methods	This work
pGPH8879	Φ(<i>malT'</i> - <i>lacZ</i>)1hyb in pJEL249	This work
pGPH8881	Φ(<i>malT'</i> - <i>lacZ</i> ⁺)1 in pJEL250	This work
pGPH9260	Φ(<i>malTp1 malTp10'</i> - <i>lacZ</i> ⁺)1 in pJEL250	This work
pGPH9261	Φ(<i>ΔmalT'</i> - <i>lacZ</i> ⁺)2 in pJEL250	This work
pGPH9463	Φ(<i>malP'</i> - <i>lacZ</i> ⁺)1 in pJEL250	This work

at a position 1 to 23 bp from the start of the *malT* transcript. The expression of the maltose regulon is induced when maltose or maltodextrins are present in the growth medium (exogenous induction). Low un-induced expression is controlled by the internal synthesis of inducers (endogenous induction) and is strongly repressed at high osmolarity. The induced level of maltose regulon expression is not affected by osmolarity (Bukau *et al.*, 1986). In addition to its role in transport, MalK influences maltose gene expression, probably by competing with the endogenous inducer for MalT_i. Decker *et al.* (1993) postulated that in the absence of MalK, MalT would not require an inducer to be active.

Here, we describe the effects of pH₀ on the activity of the *malEp*, *malKp*, *malPp*, *malSp* and *malTp* promoters and demonstrate that pH₀ modulation of MalT-dependent genes is a direct consequence of *malT* pH₀ modulation. We provide evidence that the cAMP-CRP complex is involved in pH₀-dependent *malTp* activity.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. Standard media were used in this study, including LB broth (Miller, 1992), MacConkey (MC) (Difco) and M63 minimal medium (MM; Miller, 1992) supplemented with 3 µM thiamine hydrochloride, the appropriate amino acids and various sugars as carbon source. Buffered MM contained 50 mM MES, 50 mM TAPS, 40 mM KCl, 21 mM NH₄Cl, 100 µM CaCl₂, 1 mM MgCl₂, 0.7 µM FeCl₃, 25 µM Na₂SO₄, 1 mM KH₂PO₄ and 50 mM NaCl (Heyde *et al.*, 1991). These media were adjusted with 1 M NaOH or 1 M HCl to pH 4.75, 5, 5.25, 5.5, 5.75, 6.5, 7, 7.5 and 8. An osmolarity of about 340 mOsm for MM was determined by measuring the freezing point with an osmometer (Fiske type OS/220). MM was supplemented with 3 µM thiamine hydrochloride and 44 mM glycerol as carbon source. Maltose (58 mM) was added to this basal MM to induce the maltose regulon. When required, antibiotics were added at the following concentrations: 100 µg ampicillin ml⁻¹; 50 µg kanamycin ml⁻¹; 15 µg tetracycline ml⁻¹; 30 µg ampicillin ml⁻¹ for pJEL derivatives.

Growth conditions. Overnight subcultures, grown in LB at 30 °C, were used for inoculating MM adjusted to different pH₀

values and supplemented with glycerol (basal medium) or glycerol and maltose (inducer medium). These cultures were incubated at 30 °C until stationary phase was reached. At this point, the different buffered cultures were diluted with the same fresh medium to an OD₆₀₀ of 0.02 (path length 1 cm, Jouan spectrophotometer). These new cultures were incubated at 30 °C up to an OD₆₀₀ between 0.2 and 0.3. Up to this cell density, the pH of the growth medium remains constant. Five millilitre cultures were grown in sterile culture tubes (20 mm diam.) and aerated in a gyratory water bath at 200 r.p.m.

Enzyme assays. β-Galactosidase activities associated with operon or gene fusions were measured on toluenized cells as described previously (Heyde & Portalier, 1987). One unit (U) of β-galactosidase activity was defined as the amount of enzyme that hydrolysed 1 nmol substrate min⁻¹. Independent experiments were performed and results were averaged to obtain the indicated values. Error bars display the SD. The absence of error bars indicates that the SD fell below the resolution limit of the graphing program.

In vitro plasmid construction. Plasmid DNA extraction, restriction endonuclease analysis, cloning, transformation and Southern blotting procedures were as described by Maniatis *et al.* (1982). All constructions in pJEL plasmid vectors were controlled by plasmid DNA sequencing, carried out using the dideoxy chain-termination method and the Oncor Fidelity kit (Appligene), according to the manufacturer's instructions. pGPH1767 and pGPH1768 are pJEL250 derivatives containing, respectively, *malEp* and *malKp* promoters of the *malB* region. Cloning of the 481 bp *EcoRI* fragment from pOM18 (Raibaud *et al.*, 1989) into the *EcoRI* site and in both orientations resulted in the (*malE'*-*lacZ*⁺)1 and (*malK'*-*lacZ*⁺)1 operon fusions. pGPH9463 and pGPH8881 are pJEL250 derivatives containing, respectively, *malPp* and *malTp* promoters of the *malA* region. Cloning of the 811 bp *EcoRI* fragment from pOM12 (Chapon & Kolb, 1983) into the *EcoRI* site and in both orientations resulted in the (*malP'*-*lacZ*⁺)1 and (*malT'*-*lacZ*⁺)1 operon fusions. Insertion of the same 811 bp fragment in-frame with *lacZ* into the *EcoRI* site of pJEL249 gave pGPH8879 which harbours an active (*malT'*-*lacZ*)1hyb gene fusion. pGPH9261 is a pJEL250 derivative containing a minimal *malTp* promoter deleted for the upstream region. Cloning of a blunt-ended 560 bp *SalI*-*Clal* fragment from pOM2 (Raibaud *et al.*, 1985) into the blunt-ended *EcoRI* site of pJEL250 gave the (*ΔmalT'*-*lacZ*⁺)2 operon fusion. pGPH8579 contains the whole *malB* region, except the 3' end

of the *malM* gene which was inserted as a *Bam*HI-*Pvu*II fragment into plasmid pBR328. This 12013 bp recombinant plasmid carries the ampicillin resistance gene. pGPH8579K is a derivative of pGPH8579 with a kanamycin resistance cassette (Genblock, Pharmacia) inserted into the *Eco*RI site of the *malK* gene.

In vivo genetic methods. Generalized transduction with P1_{vir} was performed according to Miller (1992). The (*malTp1 malTp10-lacZ*⁺)1 operon fusion was constructed by allelic exchange between the chromosome of pop4127 (*malTp1 malTp10 Δcrp Δlac*) and the plasmid-borne (*malT'-lacZ*⁺)1 operon fusion. pop4127 was infected by P1 lysate and propagated on the GPH8881 strain (*Δlac*) which harboured the plasmid-borne (*malT-lacZ*⁺)1 operon fusion. Lac⁺ Amp^R colonies were selected. They were unable to metabolize maltose. These colonies result from the transfer of the plasmid into pop4127 and subsequent allelic exchange between chromosomal *malTp1 malTp10* and wild-type plasmid-borne *malT* promoters. Indeed, plasmid-borne *lacZYA* could be expressed in the *Δcrp* background (pop4127) only if the *malTp1 malTp10* promoter was substituted to the *malT* promoter on the plasmid. Moreover, the chromosomal *malT* gene cannot be expressed in the *Δcrp* background. The strain became Mal⁻ if the wild-type *malT* promoter was substituted to the *malTp1 malTp10* promoter. A plasmid harbouring the (*malTp1 malTp10'-lacZ*⁺)1 operon fusion was named pGPH9260. The presence of the *malTp10* mutation was checked by the presence of a new *Eco*RI site introduced by the mutation. Then, competent cells of strain MC4100 were transformed with pGPH9260 resulting in strain GPH9275. The inactivation of the chromosomal *malK* gene was done with the help of pGPH8579K. This plasmid, which carries *malK'-Kan^R-malK*, was linearized with *Eag*I restriction endonuclease and then used to transform electro-competent cells of TE2680. Kan^R white clones were selected on MC maltose kanamycin medium. In these clones, the chromosomal *malK* gene was interrupted by the kanamycin cassette. This construct was then transferred into the MC4100 strain by P1 transduction. The correct insertion of the cassette was confirmed by Southern blotting.

RESULTS

Expression of the maltose regulon is pH₀-modulated

Previous studies have shown that *malB* expression is pH₀-regulated (Heyde & Portalier, 1987; Heyde *et al.*, 1991). To determine if the pH₀ effect operates at the transcriptional level on *malA* and *malB* operons, *lac* operon fusions with *malPp*, *malKp* and *malEp* were constructed *in vitro* using pJEL250 (Valentin-Hansen *et al.*, 1986; see Methods). A chromosomal *malS-lacZ* operon fusion (Freundlieb & Boos, 1986) was also analysed. The use of the temperature-sensitive pJEL250 replicon allowed us to maintain *malp-lacZ* fusion copy numbers similar to chromosomal maltose promoters, thus minimizing a possible titration of regulatory factors by excess copies of reporter constructs. Moreover, the use of plasmid operon fusions, which maintained a functional maltose regulon in the cell, prevented the alteration of more subtle regulatory circuits.

Fig. 1 shows that growth in MM at neutral or higher pH₀ stimulated transcription initiated at *malKp* (Fig. 1a, b), *malEp* (c, d), *malPp* (f) and *malSp* (g) in the absence or presence of maltose, but did not stimulate the

expression of the *ompA* gene (e) which was previously reported to be pH₀-independent (Heyde *et al.*, 1991). The pH₀ regulation of maltose genes also led to variations in the concentrations of LamB (Heyde & Portalier, 1987) and MalQ (data not shown). Depending on promoters and growth conditions, *R* values, which correspond to the ratio between β-galactosidase activity during growth at pH₀ 6.5 and 4.75, varied from 3 to 26 (Fig. 1). Uninduced (basal) *malKp* activity displayed an *R* value of 8 ± 3 , higher than the *R* value for induced *malKp* activity (3.5 ± 0.5) (compare Fig. 1a with 1b). Finally, as both basal and induced activities of maltose promoters depend on pH₀, variation in the level of endogenous inducer concentration as a function of pH₀ could not alone explain the pH₀ regulation of maltose genes.

Effect of pH₀ on *malT* regulatory gene expression

Since the expression of all maltose promoters requires activation by the MalT regulatory protein, we first tested if the pH₀ regulation of the maltose regulon was the mere consequence of pH₀-dependent *malTp* activity. *lac* operon or gene fusions with *malTp* were constructed *in vitro* using pJEL250 or pJEL249, respectively (see Methods). Fig. 2(a) and (b) show that the expression of (*malT'-lacZ*⁺)1 operon and (*malT'-lacZ*)1hyb gene fusions was stimulated during growth in MM at neutral or higher pH₀, with *R* values ranging from 3 ± 0.5 to 6 ± 2.4 . *R* values obtained with operon and gene fusions were not significantly different. These results indicate that during growth in MM, *malT* expression is predominantly pH₀-regulated at the transcriptional level. We previously showed that during growth in LB medium, *malT* expression remains constant whatever the pH₀ (Heyde *et al.*, 1991). We re-examined *malT* expression during growth in buffered LB medium and found that it was pH₀-regulated with an *R* value of 2. Therefore, *malT* pH₀ regulation was not specific to growth in MM. For pH₀ values lower than 5.75, we observed a strong correlation between the increase in *malTp* activity and *malKp*, *malEp* and *malPp* activities (compare Fig. 2a to Fig. 1). For pH₀ values higher than 5.75, the increase of *malTp* activity contrasted with decreased or steady activity of maltose promoters. The correlation between *malTp* and *malKp* activities for pH₀ values lower than 5.75 strongly suggests that the pH₀-dependent *malTp* activity is responsible for pH₀ modulation of maltose gene expression.

Characterization of *malT* pH₀ modulation

malT gene transcription requires the binding of the cAMP-CRP complex on *malTp* (Chapon & Kolb, 1983) and is repressed by the binding of Mlc (Decker *et al.*, 1998). The effect of DNA deletion upstream of *malT* was analysed with the construction of a (*ΔmalT'-lacZ*⁺)2 operon fusion in which DNA upstream of position -120 relative to the start of transcription was lacking (Fig. 3a). The effect of an *mlc::Tn10Kan* insertion on *malT* transcription was analysed with the (*malT'-lacZ*⁺)1 operon fusion (Fig. 3a). Data from these

Fig. 1. Influence of pH₀ on the transcription of maltose genes. β -Galactosidase activity was assayed during growth at 30 °C in MM adjusted to different pH values and supplemented with glycerol (a, c and e) or glycerol and maltose (b, d, f and g), and ampicillin for plasmid-containing strains. Data are the mean values ($\pm$ SD) from at least three independent experiments. R values correspond to the ratios between β -galactosidase activities at pH₀ 6.5 and 4.75. *malS* and *malP* expression was not assayed in the absence of maltose because basal expression was not detected. (a, b) $(malK'-lacZ^+)$ 1 operon fusion on pJEL250 (GPH1768). (c, d) $(malE'-lacZ^+)$ 1 operon fusion on pJEL250 (GPH1767). (e) $(ompA::lacZ)_{hyb}$ chromosomal gene fusion (GPH8805). (f) $(malP'-lacZ^+)$ 1 operon fusion on pJEL250 (GPH9463). (g) $(malS'-lacZ^+)$ 1 chromosomal operon fusion (SF1707).

experiments showed that *malT* transcription is increased in the absence of Mlc compared to the wild-type strain but is still pH₀-regulated (Fig. 3a). To elucidate the role of cAMP-CRP in *malT* pH₀ modulation, both *malTp1* and *malTp10* mutations were introduced, by *in*

in vivo recombination, into the plasmid-borne $(malT'-lacZ^+)$ 1 operon fusion to produce the $(malTp1 malTp10-lacZ^+)$ 1 operon fusion (see Methods). The *malTp1* mutation makes the -10 sequence closer to the consensus TATAAT box, allowing constitutive *malT*

Fig. 2. Influence of pH₀ on *malT* gene expression. β -Galactosidase activity was assayed during growth at 30 °C in MM adjusted to different pH values and supplemented with glycerol and ampicillin. Data are the mean values ($\pm$ SD) from at least three independent experiments. R values were calculated as indicated in the legend to Fig. 1. (a) (*malT'*-*lacZ'*)1 operon fusion on pJEL250 (GPH8881). (b) (*malT'*-*lacZ'*)1hyb gene fusion on pJEL249 (GPH8879).

transcription in the absence of the cAMP-CRP complex (Chapon, 1982). The *malTp10* mutation alters the CRP binding site in *malTp* and prevents the fixation of the cAMP-CRP complex (Raibaud *et al.*, 1991). Data from Fig. 3(b) show that growth in basal MM at low pH₀ did not repress transcription initiated at *malTp1 malTp10*, leading to high pH₀-independent expression of *malT*. These results imply that (i) the DNA upstream of -120 and Mlc do not contribute to the repression of *malT* expression during growth in basal MM at low pH₀ and (ii) the cAMP-CRP complex is required for the pH₀ modulation of *malT* transcription. To test the role of cAMP in *malT* gene pH₀ modulation, *malT* transcription was analysed in a Δ *cya crp** genetic background. The *crp** mutation affects the coding sequence of *crp* and makes the CRP* protein able to activate cAMP-CRP-dependent promoters even in the absence of cAMP (Harman *et al.*, 1986). Fig. 3(b) shows that in the Δ *cya*

Fig. 3. Genetic analysis of *malT* pH₀ regulation. β -Galactosidase activity was assayed during growth at 30 °C in MM adjusted to different pH values and supplemented with glycerol and ampicillin. Data are the mean values ($\pm$ SD) from at least three independent experiments. R values were calculated as indicated in the legend to Fig. 1. Data for the wild-type strain are those presented in Fig. 2(a). (a) (Δ *malT'*-*lacZ'*)2 operon fusion on pJEL250 in wild-type background (GPH9274, ○). (*malT'*-*lacZ'*)1 operon fusion on pJEL250 in the *mlc::Tn10Kan* (GPH9949, ▲) or wild-type (GPH8881, +) genetic background. (b) (*malTp1 malTp10'*-*lacZ'*)1 operon fusion on pJEL250 in wild-type background (GPH9275, □). (*malT'*-*lacZ'*)1 operon fusions on pJEL250 in a Δ *cya crp** (GPH8892, ♦) or wild-type (GPH8881, +) genetic background. The expression of *malT* in a Δ *cya crp** derivative could not be reported at pH₀ 4.75 because this derivative grew very poorly at this pH₀.

*crp** derivative, *malT* transcription is less affected by pH₀. This result indicates that pH₀ regulation of *malT* may be in part related to either pH₀ modulation of the cAMP pool or conformational changes in CRP induced by cAMP binding.

pH₀ modulation of *malK* transcription is a consequence of the pH₀ regulation of *malT*

As the pH₀ modulation of the plasmid-borne (*malTp1 malTp10'*-*lacZ'*)1 operon fusion was abolished (Fig. 3b), the pH₀ regulation of the plasmid-borne (*malK'*-

Fig. 4. Influence of the *malTp1 malTp10* mutations on *malK* pH₀ regulation. β -Galactosidase activity was assayed during growth at 30 °C in MM adjusted to different pH values and supplemented with glycerol and ampicillin. Data are the mean values ($\pm$ SD) from at least two independent experiments. Data for the wild-type strain are those presented in Fig. 1(a). (*malK'*-*lacZ*⁺)1 operon fusion on pJEL250 in a *malTp1 malTp10* (GPH8818, $\square$) or wild-type (GPH1768, $\blacksquare$) genetic background.

lacZ⁺)1 operon fusion in the presence of chromosomal *malTp1 malTp10* mutations was measured. Data in Fig. 4 show that transcription initiated at *malKp* was no longer pH₀-regulated in the *malTp1 malTp10* genetic background. At low pH₀, transcription was stimulated and reached the transcription level observed in the wild-type strain during growth at high pH₀ (specific activity of 400). This result clearly demonstrates that the pH₀-dependent activity of *malKp* is a direct consequence of *malT* pH₀ regulation. Thus, pH₀ control of MalT_i synthesis produces subsequent higher amounts of MalT_a at high than at low pH₀.

MalT_i/MalT_a equilibrium and pH₀-dependent expression of *malK*

The expression of the (*malK'*-*lacZ*⁺)1 operon fusion was measured in the presence of the *malT*^c, *malK* or *malQ* null mutation. These mutations were previously shown to shift MalT_i/MalT_a equilibrium towards MalT_a. The *malT*^c mutation affects the *malT* coding sequence. It results in the synthesis of a MalT_i protein with an increased affinity for the inducer and some activity in the absence of maltotriose (Dardonville & Raibaud, 1990). In such a background (Fig. 5a), the expected (*malK'*-*lacZ*⁺)1 basal overexpression was observed (specific activity of 2000) and *malKp* activity was constant whatever the pH₀. The induced expression of *malK*, controlled by MalT^c, was identical to uninduced expression (Fig. 5a and b), but was lower than the maximal induced expression (specific activity of 6000) observed in the parental strain (Fig. 5b).

The MalQ protein has been shown to remove the endogenous inducer derived from glycogen (Bukau *et*

al., 1986). In the absence of MalQ, the higher amount of endogenous inducer available leads to an increase in the amount of MalT_a. Using a previously described *malQ* null allele, we have shown that, in the absence of MalQ (Fig. 5c), expression of the (*malK'*-*lacZ*⁺)1 operon fusions was unusually high and appeared to be less affected by pH₀ ($R = 2 \pm 0.2$ versus 8 ± 3 for the parental strain).

In the absence of MalK, the basal expression of the maltose regulon is strongly increased. A putative function of MalK would be to compete with the endogenous inducer for binding to MalT_i (Decker *et al.*, 1993), leading to lower amounts of MalT_i available for conversion into MalT_a. In the absence of MalK (Fig. 5d), the expected (*malK'*-*lacZ*⁺)1 overexpression was observed, but this expression was still pH₀-dependent ($R = 8.4 \pm 2.5$ versus 8 ± 3 for the parental strain).

Induced conditions and *malT*^c, *malQ* or *malK* mutations have all been shown to shift the MalT_i/MalT_a equilibrium towards MalT_a. In the presence of MalT^c (*malT*^c mutation) or without MalQ (*malQ* mutation), *malK* expression more or less depends on pH₀. However, without MalK (*malK* mutation) or in the presence of exogenous inducer (Fig. 1b), *malK* expression depends on pH₀ as in the parental strain in uninduced conditions. This discrepancy between data obtained with conditions known to shift MalT_i/MalT_a equilibrium towards MalT_a indicates that the increase in MalT_i conversion would not be the only effect of some of these mutations or induced conditions.

DISCUSSION

Our results show that during growth in MM, *malTp* activity is pH₀-dependent, which led to a *malT* transcription level three times higher at medium pH₀ than at low pH₀. This regulation depends on the binding of cAMP-CRP to *malTp* and results in the pH₀ modulation of basal and induced activity of MalT-dependent promoters (*malKp*, *malEp*, *malPp* and *malSp*). Accordingly, the amount of MalT_i produced would depend on pH₀ and the amount of MalT_a would be higher at high pH₀ than at low pH₀. Bukau *et al.* (1986) reported the osmoregulation of maltose genes during growth in MM. However, molecular mechanisms operating in osmoregulation are different from those involved in pH₀ regulation. Indeed, *malT* expression does not depend on medium osmolarity and only the basal expression of maltose genes is osmodependent. Moreover, the cAMP-CRP complex is not required for the osmoregulation of maltose genes. Ehrmann & Boos (1987) postulated that osmoregulation is mediated by regulation of the synthesis of two endogenous inducer classes, one made under low osmolarity and the other under high osmolarity. Our results do not support the idea of pH₀-dependent synthesis of an endogenous inducer. Indeed, pH₀ regulation works when an exogenous inducer is provided and is not effective as soon as MalT synthesis does not fluctuate with the pH₀ because of *malTp1 malTp10* mutations. On the basis of

Fig. 5. Influence of *maltT1*, *malQ::Tn10* and *malK::Kan^R* mutations on *malK* pH_0 regulation. β -Galactosidase activity was assayed during growth at 30 °C in MM adjusted to different pH values and supplemented with ampicillin and glycerol (a, c and d) or glycerol plus maltose (b). Data are mean values ($\pm$ SD) from at least two independent experiments. Data for the wild-type strain are those presented in Fig. 1(a) and (b). *R* factors were calculated as indicated in the legend to Fig. 1. (a, b) (*malK'-lacZ'*)₁ operon fusion on pJEL250 in a *maltT1* (GPH8561, $\square$) or wild-type (GPH1768, $\blacksquare$) genetic background. (c) (*malK'-lacZ'*)₁ operon fusion on pJEL250 in a *malQ::Tn10* (GPH8888, $\circ$) or wild-type (GPH1768, $\blacksquare$) genetic background. (d) (*malK'-lacZ'*)₁ operon fusion on pJEL250 in a *malK::Kan^R* (GPH8863, $\circ$) or wild-type (GPH1768, $\blacksquare$) genetic background.

our results, we propose that in the wild-type strain during growth in MM, the pH of the external medium influences *malT* transcription through the action of the cAMP-CRP complex. Indeed our observations regarding the deleted *malTp* promoter demonstrate that the minimal *malT* promoter contains all the regulatory regions for establishing the pH_0 regulation. Moreover, Mlc binding to its operator binding site was not required by the regulatory circuit leading to pH_0 regulation of maltose regulon.

It should be emphasized that most of the pH_0 -regulated genes presently described are cAMP-CRP dependent (Olson, 1993). pH_0 modulation of the amount of cAMP-CRP is unlikely. Indeed, *cya* and *crp* expression and total cAMP do not depend on pH_0 (Coll *et al.*, 1994 and

data not shown). Moreover, all cAMP-CRP-dependent genes are not pH_0 -dependent. We propose that pH_0 may indirectly affect cAMP-CRP binding at *malTp*, either by pH_0 -induced alterations of *malTp* topology or by the presence of a pH_0 -dependent effector that may act in concert with cAMP-CRP. As *malTp* activity depends less on pH_0 in the Δcya *crp** derivative (Fig. 3b), the CRP* protein may be locked in a configuration that is less sensitive to DNA topology alterations or to interactions with the putative coregulator. Under inducing conditions, an *R* value of 3 for *malTp* leads to an *R* value of 26 for *malSp* activity. This result could reveal some different features in MalT binding at *malSp* or some function of the periplasmic α -amylase (MalS) in pH_0 regulation. Indeed, the *malS* gene is inactivated by the fusion we used in this study.

Further characterization of *malTp* activation by cAMP-CRP aimed at the understanding of the pH₀ regulation of maltose genes should help to define the mechanisms able to sense and transduce the pH₀ stimulus.

ACKNOWLEDGEMENTS

We are obliged to Evelyne Richet and Winfried Boos for providing strains, stimulating discussions and critical reading of the manuscript. We thank M. Berlyn, T. Elliott and P. Valentin-Hansen for providing strains and plasmids. We thank A. Garel for primer synthesis and B. Dequatre for excellent technical assistance. S.A. was supported by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche. This work was supported by research grants from the Centre National de la Recherche Scientifique (UMR 5534) and the University Claude Bernard Lyon I.

REFERENCES

- Bachmann, B. J. (1990). Linkage map of *Escherichia coli* K-12, edition 8. *Microbiol Rev* **54**, 130–197.
- Boos, W., Peist, R., Decker, K. & Zdych, E. (1996). The maltose system of *Escherichia coli*. In *Regulation of Gene Expression in Escherichia coli*, pp. 201–229. E. C. C. Lin & A. S. Lynch. Austin, TX: R. G. Landes.
- Bukau, B., Ehrmann, M. & Boos, W. (1986). Osmoregulation of the maltose regulon in *Escherichia coli*. *J Bacteriol* **166**, 884–891.
- Chapon, C. (1982). Role of the catabolite activator protein in the maltose regulon of *Escherichia coli*. *J Bacteriol* **150**, 722–729.
- Chapon, C. & Kolb, A. (1983). Action of CAP on the *malT* promoter *in vitro*. *J Bacteriol* **156**, 1135–1143.
- Coll, J. L., Heyde, M. & Portulier, R. (1994). Expression of the *nmpC* gene of *Escherichia coli* K-12 is modulated by external pH. Identification of *cis*-acting regulatory sequences involved in this regulation. *Mol Microbiol* **12**, 83–93.
- Dardonville, B. & Raibaud, O. (1990). Characterization of *malT* mutants that constitutively activate the maltose regulon of *Escherichia coli*. *J Bacteriol* **172**, 1846–1852.
- Decker, K., Peist, R., Reidl, J., Kossmann, M., Brand, B. & Boos, W. (1993). Maltose and maltotriose can be formed endogenously in *Escherichia coli* from glucose and glucose-1-phosphate independently of enzymes of the maltose system. *J Bacteriol* **175**, 5655–5665.
- Decker, K., Plumbridge, J. & Boos, W. (1998). Negative transcriptional regulation of a positive regulator; the expression of *malT*, encoding the transcriptional activator of the maltose regulon of *Escherichia coli*, is negatively controlled by Mlc. *Mol Microbiol* **27**, 381–390.
- Ehrmann, M. & Boos, W. (1987). Identification of endogenous inducers of the *mal* regulon in *Escherichia coli*. *J Bacteriol* **169**, 3539–3545.
- Elliott, T. (1992). A method for constructing single-copy *lac* fusions in *Salmonella typhimurium* and its application to the *hemA-prfA* operon. *J Bacteriol* **174**, 245–253.
- Freundlieb, S. & Boos, W. (1986). α -Amylase of *Escherichia coli*, mapping and cloning of the structural gene, *malS*, and identification of its product as a periplasmic protein. *J Biol Chem* **261**, 2946–2953.
- Harman, J. G., McKenny, K. & Petrekofsky, A. (1986). Structure function analysis of three cAMP-independent forms of the cAMP receptor protein. *J Biol Chem* **261**, 16332–16339.
- Heyde, M. & Portulier, R. (1987). Regulation of major outer membrane porin proteins of *Escherichia coli* K-12 by pH. *Mol Gen Genet* **208**, 511–517.
- Heyde, M., Coll, J. L. & Portulier, R. (1991). Identification of *Escherichia coli* genes whose expression increases as a function of external pH. *Mol Gen Genet* **229**, 197–205.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Olson, E. R. (1993). Influence of pH on bacterial gene expression. *Mol Microbiol* **8**, 5–14.
- Raibaud, O. & Richet, E. (1987). Maltotriose is the inducer of the maltose regulon of *Escherichia coli*. *J Bacteriol* **169**, 3059–3061.
- Raibaud, O., Gutierrez, C. & Schwartz, M. (1985). Essential and nonessential sequences in *malPp*, a positively controlled promoter in *Escherichia coli*. *J Bacteriol* **161**, 1201–1208.
- Raibaud, O., Vidal-Ingigliardi, D. & Richet, E. (1989). A complex nucleoprotein structure involved in activation of transcription of two divergent *Escherichia coli* promoters. *J Mol Biol* **205**, 471–485.
- Raibaud, O., Vidal-Ingigliardi, D. & Kolb, A. (1991). Genetic studies on the promoter of *malT*, the gene that encodes the activator of the *Escherichia coli* maltose regulon. *Res Microbiol* **142**, 937–942.
- Richet, E. & Raibaud, O. (1989). MalT, the regulatory protein of the *Escherichia coli* maltose system, is an ATP-dependent transcriptional activator. *EMBO J* **8**, 981–987.
- Schwartz, M. (1987). The maltose regulon. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1482–1502. Edited by F. C. Neidhardt, J. L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- Valentin-Hansen, P., Albrechtsen, B. & Løve Larsen, J. E. (1986). DNA-protein recognition: demonstration of three genetically separated operator elements that are required for repression of the *Escherichia coli* *deoCABD* promoters by the DeoR repressor. *EMBO J* **5**, 2015–2021.
- Zilberstein, D., Agmon, V., Schuldiner, S. & Padan, E. (1984). *Escherichia coli* intracellular pH, membrane potential and cell growth. *J Bacteriol* **158**, 246–252.

Received 16 February 1998; revised 22 July 1998; accepted 17 August 1998.