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Evidence for two promoters upstream of the *pts* operon: Regulation by the cAMP receptor protein regulatory complex

(phospho*enol*pyruvate:glycose phosphotransferase system/sugar transport/bacterial transcription)

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ABSTRACT **Several potential target sites for multiple regulatory mechanisms were previously identified in the 5′ flanking region of the *pts* operon. We have investigated the *in vitro* interactions of the cAMP receptor protein (CRP)·cAMP regulatory complex with two DNA binding sites, by gel mobility-shift assays, and report the analysis of the functional role of each of the binding sites *in vivo*. Promoter–reporter gene fusion studies identified two CRP·cAMP-dependent promoters (the previously identified P_1 and another promoter, P_0) upstream of *ptsH*. The *crr* promoters (P_2) within *ptsI* may be negatively regulated by CRP·cAMP.**

The phospho*enol*pyruvate:glycose phosphotransferase system (PTS) catalyzes the phosphorylation and transport of its sugar substrates and acts as a major signal-transduction system in bacterial cells. Thus, the PTS must be responsive to multiple and diverse external signals (for a review, see ref. 1).

The PTS has been extensively studied in *Escherichia coli* and *Salmonella typhimurium* and consists of a complex array of reversibly phosphorylated cytoplasmic and membrane proteins. Two general, cytoplasmic proteins are required for the phosphorylation of all PTS substrates: enzyme I, encoded by *ptsI*, and the histidine-containing phosphoprotein HPr, encoded by *ptsH*. Specific membrane complexes are required for translocation of the individual PTS sugars, and one of these, the glucose-specific complex, comprises a soluble protein (III^{Glc}) and a membrane-bound enzyme (IIB^{Glc}) encoded by the *crr* and *ptsG* genes, respectively.

The *ptsH*, *ptsI*, and *crr* genes constitute an operon (Fig. 1) that is located at 52 min on the *E. coli* chromosome; *ptsG* is unlinked at 25 min. The PTS, itself, is stringently regulated and the mechanisms underlying the genetic regulation of *pts* expression are unclear and apparently complex. For example, growth of wild-type cells on PTS sugars, such as glucose, induces up to 3-fold increased activities of enzyme I, HPr, and III^{Glc} (2–4) and, as shown below, some of the *pts* promoters respond positively to the cAMP receptor protein (CRP)·cAMP complex, although glucose lowers the level of cAMP.

The complete DNA sequence of *ptsH*, *ptsI*, *crr* and their flanking regions suggested two canonical binding regions for CRP·cAMP upstream of the first gene, *ptsH*, and a promoter for *crr*, the last gene, within and toward the 3′ terminus of *ptsI* (5). Transcriptional analysis of the region (4) suggested one promoter upstream of *ptsH*, designated P_1, and two within *ptsI* that regulated transcription of *crr*, designated P2-I and P2-II. The designations P_1 and P_2 will be employed in this paper for the previously described promoters.

In the present studies, we have measured CRP binding to the two putative binding sites upstream of *ptsH* by gel mobility-shift assays. We also report CRP·cAMP-independent and CRP·cAMP-dependent promoter activities in the DNA regions preceding and within the *pts* operon, including another promoter, designated P_0, upstream of P_1.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* host strains used in this study were SA2600 (wild type) (F⁻ *his rpsL relA*) and isogenic strains derived from SA2600, SA2426 (Δ*cya*) (F⁻ *his rpsL relA* Δ*cya854 trp*::Tn*10relA*), G792 (Δ*cyacrp592**) (F⁻ *his rpsL relA* Δ*cya854 crp*592 trp*::Tn*10relA*), G1130 (Δ*crp*) (F⁻ *his rpsL relA ilv*::Tn*10*Δ*crpBS990*), and G806 (Δ*cya*Δ*crp*) (F⁻ *his rpsL relA* Δ*cya854* Δ*crpBS990 trp*::Tn*10relA*) (6).

Plasmid Construction. DNA fragments (see Fig. 1) were generated by digestion of pDS20 (5) with the restriction enzymes (GIBCO/BRL) *Hin*dIII and *Xho* I (HX) or *Apa*LI and *Acc* I (AA). The HX fragment was subsequently digested with *Cla* I to yield HC and CX fragments. All fragments were purified by agarose gel electrophoresis. DNA was extracted from agarose gel slices by using Geneclean (Bio 101, La Jolla, CA) or Prep-A-Gene (Bio-Rad) kits. Purified fragments were ligated into the promoter-selection vector pKK232-8 (Pharmacia LKB). pKP2 contained the 91-base-pair (bp) *tac* promoter GenBlock (Pharmacia LKB) ligated into pKK232-8.

Synthesis of ^{32}P-Labeled DNA. Purified DNA fragments (see above) were radiolabeled using [α-^{32}P]dCTP (DuPont/New England Nuclear) and the Klenow fragment of DNA polymerase I (7); unincorporated nucleotide was removed by gel filtration on Centri-sep columns (Schleicher & Schuell).

DNA–Protein Binding Assay. Binding of CRP·cAMP to DNA was measured during the gel shift or gel retardation assay. CRP·cAMP and the DNA fragments of interest were incubated at 22°C for 30 min. The final incubation mixture contained 20 mM Tris·HCl (pH 7.5), 10% (vol/vol) glycerol, 50 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 40 μM cAMP and CRP, and [^{32}P]DNA. At the end of the incubation, 1 μl of sample buffer containing 1% (wt/vol) bromophenol blue, 0.1% (wt/vol) xylene cyanol, and 50% (vol/vol) glycerol was added and the entire incubation mixture was immediately loaded onto a polyacrylamide gel (4% acrylamide; acrylamide/*N*,*N*′-methylenebisacrylamide weight ratio of 60:1) and subjected to electrophoresis. All electrophoresis was done at 4°C and at 7–9 V/cm. The polyacrylamide gels were preelectrophoresed for 90 min before the samples were loaded. The electrode buffer was 10 mM Tris·HCl, pH 7.5/1 mM potassium EDTA and was continually recirculated. Electrophoresis was con-

Abbreviations: PTS, phospho*enol*pyruvate:glycose phosphotransferase system; CRP, cAMP receptor protein; CAT, chloramphenicol acetyltransferase.

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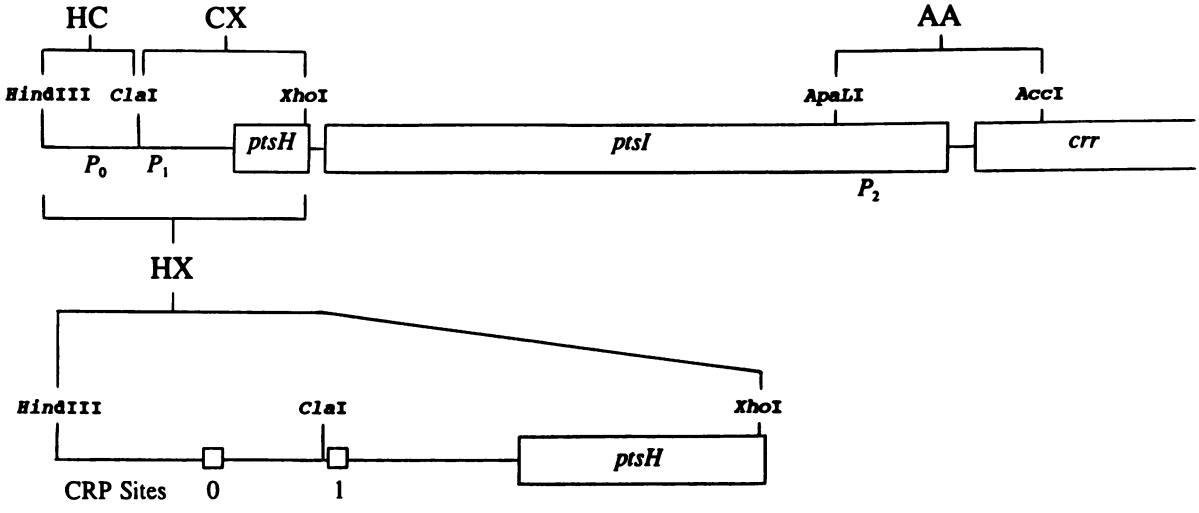

FIG. 1. Diagram of the *pts* operon. The diagram is a partial restriction map of the *ptsHI* and *crr* genes, including the HX, HC, CX, and AA cloned DNA fragments and putative promoters (P_0, P_1, and P_2). The coding regions for HPr (*ptsH*), enzyme I (*ptsI*), and III^{Glc} (*crr*) are indicated as are CRP binding sites 0 and 1.

tinued until the bromophenol blue tracking dye had migrated at least 10 cm. The polyacrylamide gel was removed from the glass plate and placed on Whatman 3 MM filter paper and dried under vacuum. Radiolabeled DNA was detected by autoradiography.

Chloramphenicol Acetyltransferase (CAT) Assay. Cells were grown at 37°C on Luria broth (8) containing ampicillin at 15 μg/ml. Inocula were grown overnight on Luria broth containing ampicillin at 15 μg/ml, and 3-ml aliquots of these cultures were added to 500 ml of medium in a 1-liter flask. Cells were harvested at midexponential phase (OD_{600} 0.6–0.8) by centrifugation at 15,000 × *g* for 15 min. Cells were resuspended in 50 mM triethanolamine·HCl, pH 7.78/1 mM EDTA/0.5 mM dithiothreitol and crude extracts were obtained as described (9). The quantity of CAT in the crude extracts was determined by immunological assay using the CAT ELISA kit (5 Prime → 3 Prime, Inc.), following the recommended procedure. The values were normalized to the amount of CAT present in the host strain carrying pKP2 (*tac* promoter).

Protein Determination. Protein present in crude extracts of cells was measured with the modified (10) procedure of Lowry.

RESULTS

CRP·cAMP Binding to *pts* DNA. To identify functional CRP·cAMP-responsive sequences in the 5′ flanking region of the *pts* operon, three DNA constructs were generated and tested for their ability to bind CRP by gel mobility-shift assays. Fig. 1 shows a schematic representation of a 746-bp *Hin*dIII–*Xho* I DNA fragment (HX) that includes the 5′ upstream region as well as almost the entire *ptsH* gene. The HX fragment, containing two putative CRP·cAMP-responsive sequences, was generated by restriction digestion of the plasmid pDS20 (5) with the enzymes *Hin*dIII and *Xho* I. To further localize these two sequences, the HX fragment was digested with *Cla* I, yielding HC and CX fragments, each containing a potential CRP binding site. When CRP·cAMP was incubated with radiolabeled HX DNA and the mixture was electrophoresed in a 4% polyacrylamide gel (Fig. 2*A*), two species of CRP-bound DNA were observed; a compact, narrow band migrated more slowly than a more diffuse band. These results are consistent with the presence of two binding sites on the HX fragment.

Direct evidence for this conclusion was obtained by isolating two fragments from HX, each containing a putative CRP binding site. The results with the two fragments, HC and CX, are also shown in Fig. 2, and it is evident that each fragment binds CRP. In these experiments, all three fragments, HX, HC, and CX, were titrated with increasing concentrations of CRP, and whereas large shifts were observed at the lowest concentration tested (10 nM) with HC (Fig. 2*C*), substantially higher levels were required to obtain similar effects with CX (Fig. 2*B*). Thus, we conclude that CRP can bind to each of the putative sites, but that the site within HC has a much higher affinity than that in CX. It is possible that the lower affinity of the CX fragment for CRP·cAMP is due to the close proximity of the CRP site to the *Cla* I-generated end of the fragment. CRP did not bind to any of the DNA fragments in the absence of cAMP (data not shown).

Measurement of *pts* Promoter Activities *in Vivo*: Effect of CRP·cAMP. De Reuse and Danchin have identified a transcription initiation site for *pts* (4) and have shown that *pts* is

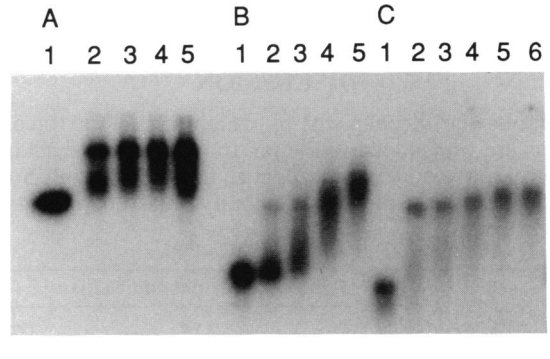

FIG. 2. Gel shift assay of CRP binding. The ^{32}P-labeled DNA probes and the electrophoresis conditions are detailed in *Materials and Methods*. The autoradiogram is from a representative experiment. (*A*) The HX fragment (4.2 nM) was incubated with CRP at 0 nM (lane 1), 10 nM (lane 2), 20 nM (lane 3), 30 nM (lane 4), and 40 nM (lane 5). (*B*) The CX fragment (6.7 nM) was incubated with CRP at 0 nM (lane 1), 10 nM (lane 2), 20 nM (lane 3), 40 nM (lane 4), and 60 nM (lane 5). (*C*) The HC fragment (9.0 nM) was incubated with CRP at 0 nM (lane 1), 10 nM (lane 2), 20 nM (lane 3), 30 nM (lane 4), 40 nM (lane 5), and 50 nM (lane 6). CRP levels are given as concentrations of dimer. cAMP (40 μM) was present in all mixtures containing CRP; no mobility shift was observed in the absence of cAMP.

positively regulated by CRP·cAMP (11). To further study the effect of CRP·cAMP on the regulation of the expression of the three genes of the *pts* operon, the potential promoters were fused to the CAT gene. In addition to the three DNA fragments described above, an *ApaLI–Acc* I fragment (AA) containing the previously identified (4) *crr* promoters (P_2) within *ptsI* (see Fig. 1) was ligated into the pKK232-8 promoter-selection vector containing a promoterless CAT gene. Transcription of the CAT gene was used for quantitative analysis of the strength of inserted promoters. pKP2, containing the strong, inducible *tac* promoter ligated into pKK232-8, was used as a control against which the activities of the other potential promoters were normalized.

The results are shown in Table 1. (*i*) In a wild-type host, the four cloned DNA fragments exhibited strong promoter activity comparable to that of the *tac* promoter. (*ii*) Although the *in vitro* experiments with fragment AA (*crr* promoter) did not show any obvious interaction with CRP, the *in vivo* results suggest that this gene may be negatively regulated by CRP·cAMP. In both a *cya* and a *crp* background, the promoter activity of DNA fragment AA increased substantially, and the addition of exogenous cAMP to the *cya* mutant depressed the promoter activity. (*iii*) In sharp contrast, HX exhibited 20–25% of its activity in wild-type cells when present in either the *cya* or the *crp* background; exogenous cAMP greatly stimulated HX promoter activity in the *cya* mutant. Thus, we conclude that CRP·cAMP positively affects the activities of one or both of the promoters in the HX fragment. (*iv*) When the two promoters in HX (HC and CX) were tested separately, however, no easily interpretable results were obtained. That is, the promoter in HC, P_0, acted as a strong promoter in a *cya* background without being affected by cAMP, and as a somewhat weaker promoter in a *crp* background. The CX (P_1) promoter did show a small positive effect by exogenous cAMP in the *cya* background but showed strong promoter activity in the *crp* background.

Thus, definitive evidence is presented for two promoters, P_0 and P_1, upstream of *ptsH*. When both promoters are located on the same fragment of DNA, HX, they are significantly and positively affected by CRP·cAMP, but when they are analyzed separately, they show no such effects. It is therefore possible that the two CRP·cAMP binding regions interact with one another. Finally, the AA fragment containing the *crr* promoters (P_2) seems relatively unaffected by CRP·cAMP.

DISCUSSION

The molecular mechanisms by which multiple extracellular signals are transduced to the *pts* transcriptional machinery, though poorly understood, appear to reflect the PTS characteristics of complexity, flexibility, and adaptability. In enteric bacteria, the level of expression of the *pts* operon changes only about 3- to 4-fold under different growth conditions (4, 11). De Reuse and Danchin (4) found that expression of *pts* was low in a Δ*cya* strain, moderate (about 2-fold higher) in a wild-type strain, and higher still (an additional 2-fold) under the same growth conditions, but with glucose or methyl α-D-glucopyranoside present.

Table 1. Promoter–CAT fusion gene activities

	Relative CAT immunoreactivity					
	Wild type		*cya*		*crp*	
Promoter	−	+	−	+	−	+
tac	1.0	1.0	1.0	1.0	1.0	1.0
HX (P_0P_1)	2.0	2.2	*0.4*	*1.3*	*0.5*	*0.5*
HC (P_0)	0.8	0.8	1.6	1.8	0.6	0.5
CX (P_1)	0.8	0.9	1.4	2.1	1.3	1.3
AA (P_2)	*1.1*	1.3	*3.1*	*1.8*	*2.7*	2.0

Promoter activity was monitored by measuring CAT reporter gene activity as described in *Materials and Methods*. Cells were incubated in the absence (−) or presence (+) of 1 mM cAMP. Values shown were normalized to the amount of CAT (μg/mg of crude extract protein) present in the host strain harboring the *tac* promoter and are averages of three determinations. Italics are used for emphasis.

In this work we have examined potential regulatory regions upstream of *ptsH*. Previously (5), we noted the presence of putative CRP binding sites at positions −174 to −154 (CRP binding site 1) and −46 to −26 (CRP binding site 2) relative to the start of transcription identified by De Reuse and Danchin (4). To be consistent with the nomenclature for the multiple promoters in the *pts* operon, we shall hereafter designate CRP binding site 1 as site 0, and binding site 2 as site 1. We report here that both CRP binding site 0 and CRP binding site 1 are indeed capable of binding CRP *in vitro*. A large DNA fragment (HX) containing both binding sites (see Fig. 1) is probably capable of binding two CRP molecules simultaneously. When a single CRP molecule is bound at either of the two sites, a diffuse band of decreased mobility relative to the DNA probe is observed in electrophoretic mobility-shift assays. The diffuse nature of the band is very likely due to the presence of two bands resulting from the binding of CRP at position 1 in the middle or at position 0 closer to one end of the HX DNA fragment; it is known that CRP-induced bending causes different degrees of retardation in gel mobility assays when CRP binds at the center of a DNA fragment rather than off-center (12). The complex with two bound CRP molecules is much more compact and is shifted to an even greater extent.

The two CRP binding sites within the HX fragment were separated into two DNA fragments, HC and CX, and each showed CRP·cAMP binding *in vitro* (mobility-shift assays); HC apparently has a higher affinity for CRP than does CX. Mobility shifts with added CRP were dependent on the presence of cAMP.

The gel shift assay results suggested that HX might contain two different promoters, and this possibility was tested by inserting DNA fragments HX, HC, CX, and AA into an appropriate vector and assaying for expression of the CAT gene. Not only did each fragment contain an active promoter, but in a wild-type host, each fragment exhibited promoter strengths that were at least comparable to that of the strong control promoter, P_{tac}.

When the plasmids were inserted into *cya* and *crp* hosts, the results indicated that CRP·cAMP positively affected the activity of the P_0 and P_1 promoters when they were within one fragment of DNA (HX). When P_0 and P_1 are separated, however, the results were not easily interpretable. Promoter activity continued to be high, but the stimulatory effects of CRP·cAMP were not as evident. The P_1 promoter showed modest stimulation by CRP·cAMP. It appears possible that the CRP·cAMP binding sites 0 and 1 may have synergistic effects on the promoters.

A transcription initiation assay by De Reuse and Danchin (4) did not identify any RNA transcripts initiating upstream of P_1. It is possible that under the growth conditions (not specified) used in those studies, P_0 does not function.

Transposon insertional analysis originally showed the presence of a *crr* promoter close to the 3′ terminus of *ptsI* (5), a result confirmed by transcriptional analysis (4). The present studies present direct evidence for the P_2 promoter(s) and also show that this promoter activity is relatively unaffected by CRP·cAMP and, in fact, may be negatively regulated by this complex.

The expression of *pts* is modulated both by CRP·cAMP and by glucose (4, 11), as well as by growth on other PTS sugars (4). The effector of glucose regulation is probably IIB^{Glc} (11). The activation by glucose is independent of CRP·cAMP

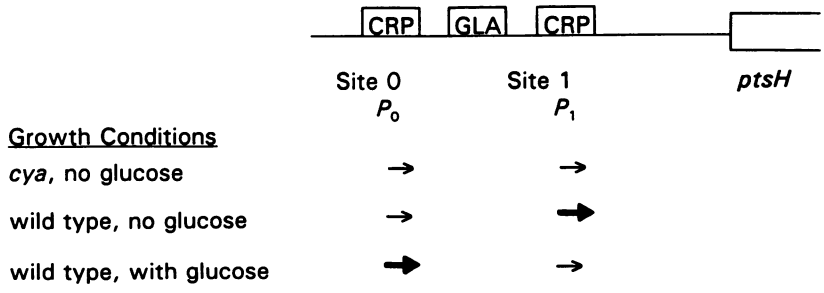

FIG. 3. A model of how *pts* can be regulated. The upper line represents the DNA of the *pts* regulatory region. P_0 is the putative promoter within the HC fragment (Fig. 1), P_1 is within the CX fragment. The CRP binding sites are indicated, as is a potential binding site for a glucose activator (GLA). In a Δ*cya* strain, transcription originates from both P_0 and P_1, at low levels. In a wild-type strain, without glucose present in the growth medium, transcription originates primarily from P_1. P_0 activity is blocked, perhaps as a consequence of the interaction between two CRP molecules bound at CRP binding sites 0 and 1. In a wild-type strain, with glucose present in the growth medium, transcription originates primarily from P_0 due to binding of a putative glucose activator. The arrows represent transcription; the thickness of the arrows indicates the magnitude of transcription.

stimulation of gene expression. Several hypotheses can be proposed to explain these results, since the mechanisms by which the CRP·cAMP complex regulates transcription are varied (for recent reviews see refs. 13 and 14). One example is shown in Fig. 3. Both P_0 and P_1 may function in a *cya* mutant that has relatively low levels of *pts* expression. When cAMP is added to the *cya* mutant, or in a wild-type strain, gene expression increases. When the growth medium does not contain glucose, growth conditions in which the cAMP concentration is high and that consequently favor CRP binding at both sites upstream of P_1, transcription initiates more frequently from P_1; P_0 expression remains low. When glucose is present in the growth medium, expression of *pts* is still higher. We propose that under these conditions transcription initiates from P_0. P_0 could be regulated by an activator that is glucose-stimulated. The activator is unknown, but since IIB^{Glc} has homology to sensors of two-component signal-transduction systems (15), this activator could be the effector of the sensor–effector pair. Under high-glucose growth conditions, there would be low CRP·cAMP levels in the cell, so P_1 would not be stimulated.

In summary, we have identified another promoter and CRP binding site for regulation of expression of *pts* and have proposed a model of how these regulatory elements could function.

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1. Meadow, N. D., Fox, D. K. & Roseman, S. (1990) *Annu. Rev. Biochem.* **59**, 497–542.
2. Stock, J. B., Waygood, E. B., Meadow, N. D., Postma, P. W. & Roseman, S. (1982) *J. Biol. Chem.* **257**, 14543–14552.
3. Mattoo, R. L. & Waygood, E. B. (1983) *Can. J. Biochem. Cell Biol.* **61**, 29–37.
4. De Reuse, H. & Danchin, A. (1988) *J. Bacteriol.* **170**, 3827–3837.
5. Saffen, D. W., Presper, K. A., Doering, T. L. & Roseman, S. (1987) *J. Biol. Chem.* **262**, 16241–16253.
6. Garges, S. & Adhya, S. (1988) *J. Bacteriol.* **170**, 1417–1422.
7. Chodosh, L. A. (1987) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Greene/Wiley-Interscience, New York), Vol. 2, pp. 12.2.2–12.2.3.
8. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
9. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A. & Roseman, S. (1982) *J. Biol. Chem.* **257**, 14461–14469.
10. Markwell, M. A. K., Maas, S. M., Bieber, H. L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210.
11. De Reuse, H. & Danchin, A. (1991) *J. Bacteriol.* **173**, 727–733.
12. Wu, H.-M. & Crothers, D. M. (1984) *Nature (London)* **308**, 509–513.
13. Adhya, S. & Garges, S. (1990) *J. Biol. Chem.* **265**, 10797–10800.
14. Botsford, J. L. & Harman, J. G. (1992) *Microbiol. Rev.* **56**, 100–122.
15. Kofoid, E. C. & Parkinson, J. S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4981–4985.