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NOTES

Involvement of Carbon Source and Acetyl Phosphate in the External-pH-Dependent Expression of Porin Genes in *Escherichia coli*

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The porin composition of the *Escherichia coli* cell envelope was analyzed during growth at different external pHs (pHo) as a function of the acetyl phosphate (AcP) level ($\Delta ackA$ pta or $ackA$ mutant, pyruvate or glucose as the carbon source) in the presence or absence of EnvZ. Our results indicate that the AcP level is influenced by the pHo, leading to modulation of the amount of OmpR-P and subsequent pHo-dependent expression of *ompF* and *ompC*. We also propose the existence of a specific signal, independent of EnvZ and AcP, leading to OmpR phosphorylation in response to pyruvate.

The outer membrane of *Escherichia coli* contains two porin proteins, OmpF and OmpC, that control the permeability of small hydrophilic molecules across the outer membrane. The total amount of OmpF and OmpC proteins is fairly constant, but their relative level varies with environmental factors, including osmolarity and external pH (pHo) (14, 15). Medium with high osmolarity or low pH favors the synthesis of OmpC, and medium with low osmolarity (LO) or high pH increases the OmpF level and reduces the OmpC level (6, 7, 19, 20). Osmoregulation of the *ompF* and *ompC* genes is mediated at the transcriptional level by the EnvZ-OmpR two-component regulatory system (2). EnvZ is the osmosensor able to sense changes in external osmolarity. It undergoes autophosphorylation at His 274 and transfers the phosphate group to Asp55 of OmpR. EnvZ also acts as an OmpR phosphate (OmpR-P) phosphatase (8). OmpR-P is a transcriptional effector of both porin genes (14). The level of OmpR-P relies on the ratio of kinase to phosphatase EnvZ activity. In vivo, the level of OmpR-P increases as osmolarity is raised (3, 16). The current genetic model for porin regulation predicts that a low level of OmpR-P stimulates the transcription of the *ompF* gene through binding to a high-affinity site and a high level of OmpR-P represses *ompF* through binding to a low-affinity site (5). *ompC* transcription is stimulated by OmpR-P through the binding to a low-affinity site (14). The high degree of homology between sensors and regulator proteins of two-component regulatory systems favors the possibility of phosphorylation of a regulator protein by a noncognate histidine kinase but also by low-molecular-weight phosphodonor molecules (21). Such cross-regulation was demonstrated to occur in vivo between CreC and PhoB (in the absence of PhoR), EnvZ and PhoB (in the absence of OmpR), and PhoB and acetyl phosphate (AcP) (in the absence of PhoR and CreC) (10, 23). EnvZ-independent mechanisms can also lead to OmpR phosphorylation and to osmolarity-dependent *ompF* expression in an *envZ* null mu-

tant (3). In vitro, OmpR was demonstrated to be phosphorylated by the noncognate histidine kinase CheA (9) and by low-molecular-weight phosphate donors such as AcP (5). In vivo experiments show that *ompF* transcription is dependent upon AcP synthesis only when EnvZ is absent (8, 11; S.-K. Kim and B. Wanner, personal communication). However, in the presence of EnvZ, the OmpC level has been demonstrated to increase as AcP accumulates (12, 18). All of these results suggest that cross-regulation would occur in vivo between OmpR and other kinase-independent, AcP-dependent mechanisms.

The role of EnvZ and OmpR-P in pHo regulation of porin expression has not yet been elucidated. Preliminary results suggest a role for EnvZ in *ompF* and *ompC* pHo regulation (19). However, in the absence of EnvZ, *ompF* expression is still pHo dependent, being higher during growth at low pHo than during growth at high pHo (6).

In this study, *ompF* and *ompC* transcription was analyzed under slightly acidic (pHo 6) or alkaline (pHo 7.8) growth conditions with glucose or pyruvate as the carbon source and in the presence of a *pta* or *ackA* mutation.

Bacterial strains, media, and growth conditions. All of the strains used are derivatives of *E. coli* K-12 and are listed in Table 1. P1 *vir* lysates and transduction experiments were performed as previously described by Miller (13). Selection and identification are indicated in Table 1.

LO minimal medium was used because, as previously shown by Thomas and Booth (19), we observed that *ompF* transcription depends on pHo only during growth in LO minimal medium. The LO minimal medium used in this study is a derivative of both the S medium described by Thomas and Booth (19) and the A medium described by Miller (13). It contained 49.2 mM KH_2PO_4-K_2HPO_4, 1 mM trisodium citrate, 0.4 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, and 3 μM thiamine hydrochloride. It was adjusted to pH 6 or 7.8 by mixing appropriate volumes of phosphate solutions (43 mM KH_2PO_4 and 6.2 mM K_2HPO_4 for pH 6 and 4.2 mM KH_2PO_4 and 45 mM K_2HPO_4 for pH 7.8). At pH 6, 40 mM NaCl was added to compensate for the change in medium osmolarity caused by the different balance of potassium and phosphate ions. Identical results

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype[a]	Source or reference[b]
BW13711	Δ*lacX74*	B. Wanner, Purdue University
BW16463	Δ*lacX74* Δ(*ackA-pta-hisQ-hisP*) *zej-223*::Tn*10*	B. Wanner, Purdue University
BW16545	Δ*lacX74 ackA200 zej-223*::Tn*10*	B. Wanner, Purdue University
Gal5	HfrP4X *thi metB lacI spoT1 relA1*	This laboratory
GPH8252	BW13711 Φ(*ompF'-lacZ*+)7.14	P1/MA2946 × BW13711 (ColA[r] Lac+)
GPH8255	GPH8252 Δ(*ackA-pta-hisQ-hisP*) *zej-223*::Tn*10*	P1/BW16463 × GPH8252 (Tet[r] Ac+/−)
GPH8257	GPH8252 *ackA200 zej-223*::Tn*10*	P1/BW16545 × GPH8252 (Tet[r] Ac+/−)
GPH8259	BW13711 Φ(*ompC'-lacZ*+)10.21	P1/MA2948 × BW13711 (Lac+)
GPH8262	SG477 *malT54*::Tn*10*	P/TST3 × SG477 (Tet[r] MeI[r])
GPH8264	GPH8252 *malT54*::Tn*10 envZ22*(Am)	P1/GPH8262 × GPH8252 (Tet[r] MeI[r])
GPH8268	GPH8252 *envZ22*(Am)	P1/Gal5 × GPH8264 (Mal+ MeI[r])
GPH8273	GPH8268 Δ(*ackA-pta-hisQ-hisP*) *zej-223*::Tn*10*	P1/BW16463 × GPH8268 (Tet[r] Ac+/−)
GPH8274	GPH8268 *ackA200 zej-223*::Tn*10*	P1/BW16545 × GPH8268 (Tet[r] Ac+/−)
GPH8277	GPH8259 *envZ22*(Am) *malT54*::Tn*10*	P1/GPH8262 × GPH8259 (Tet[r] Lac−)
GPH8280	GPH8259 *ackA200 zej-223*::Tn*10*	P1/BW16545 × GPH8259 (Tet[r] Ac−)
GPH8282	GPH8259 Δ(*ackA-pta-hisQ-hisP*) *zej-223*::Tn*10*	P1/BW16463 × GPH8259 (Tet[r] Ac−)
GPH8285	GPH8259 *envZ22*(Am)	P1/Gal5 × GPH8277 (Mal+ Lac−)
GPH8291	GPH8285 *ackA200 zej-223*::Tn*10*	P1/BW16545 × GPH8285 (Tet[r] Ac−)
GPH8293	GPH8285 Δ(*ackA-pta-hisQ-hisP*) *zej-223*::Tn*10*	P1/BW16463 × GPH8285 (Tet[r] Ac−)
JC2296	Hfr P4X *thi metB1 relA1 spoT1* Δ(*lac*)U169	This laboratory
MA2946	JC2296 Φ(*ompF'-lacZ*+)7.14	6
MA2948	JC2296 Φ(*ompC'-lacZ*+)10.21	6
MC4100	F− Δ(*lac*)U169 *araD139 rpsL relA thiA flbB*	M. Casadaban, University of Chicago
SG477	MC4100 *envZ22*(Am)	4
TST3	F− *araD139*Δ(*argF-lac*)205 *flbB5301 pstF25 relA1 rpsL150 malT54*::Tn*10 deoC1*	T. J. Silvavy, Princeton University

[a] Genetic nomenclature is from Berlyn (1).
[b] P1/A × B indicates that a P1 lysate propagated on strain A was used to transduce strain B. Transductants were selected for the first phenotype in parentheses and analyzed for the second one. Tet[r], ColA[r], and MeI[r] indicate resistance to tetracycline at 15 μg/ml−1, colicin A, and bacteriophage MeI, respectively. Lac+ or Lac−, Mal+, and Ac+/−, or Ac− indicate growth or no growth with 0.2% lactose as the carbon source, growth with 0.2% maltose, and slow or no growth with 0.4% potassium acetate, respectively.

were obtained whether 40 mM NaCl was added or not in the medium. Minimal media were supplemented with 0.04% glucose or 0.1% pyruvate, which was the carbon source. These sugar concentrations allow identical growth conditions with a doubling time of about 2 h and a concentration of about 5 × 10⁸ cells ml−1 in stationary growth phase. Overnight subcultures, grown in LB broth (13) at 30°C, were used to inoculate LO medium adjusted to different pH values. 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 optical density at 600 nm of 0.02. These new cultures were aerated and incubated at 30°C until they reached an optical density at 600 nm of 0.2 to 0.3. Until this cell density was reached, the pH of the growth medium remained constant.

Effect of carbon source on *ompF* and *ompC* pHo regulation. Strains GPH8252 and GPH8259, carrying the *ompF-lacZ* or the *ompC-lacZ* operon fusion, respectively, were grown in LO minimal medium adjusted to pH 6 or 7.8 with pyruvate or glucose as the carbon source. β-Galactosidase expression in toluene-treated cells was assayed (Fig. 1A and C and 2A and C; parent strains) as described by Miller (13). One unit of enzyme activity was defined as the amount of enzyme which hydrolyzed 1 nmol of substrate per min with 3 mM *o*-nitrophenyl-β-D-galactopyranoside as the substrate. In the parent EnvZ+ strain, with pyruvate as the carbon source, *ompF* transcription was induced 1.7-fold during growth at pHo 7.8 compared with growth at pHo 6 (Fig. 1A, parent strain) but this induction was not apparent with glucose (Fig. 1C, parent strain). Figure 2A and C show that in the parent EnvZ+ strain with pyruvate or glucose, *ompC* was induced 1.5- and 2-fold, respectively, during growth at pHo 6 compared with growth at pHo 7.8. *ompC* induction during growth at pHo 6 with glucose

or pyruvate indicates that the OmpR-P level would be higher at pHo 6 than at pHo 7.8. Identical levels of *ompF* expression at both pHos in glucose are not inconsistent with different OmpR-P levels. We assume that at pHo 6, the OmpR-P level would lead to *ompF* repression and at pHo 7.8, a lower OmpR-P level would lead to *ompF* activation, resulting in the same *ompF* expression at both pHos.

In the absence of EnvZ, with pyruvate, *ompF* expression (strain GPH8268) was slightly decreased but still induced at pHo 7.8, as in the EnvZ+ strain (compare Fig. 1B to A, parent strains). With glucose, the absence of EnvZ led to a decrease in *ompF* expression mainly at pHo 7.8. Consequently, *ompF* expression became higher at pHo 6 than at pHo 7.8 (compare Fig. 1D to C, parent strains). These results indicate that in the absence of EnvZ, *ompF* expression is still influenced by the pHo and the carbon source. With pyruvate or glucose, *ompC* was still expressed, albeit at a lower level than in the EnvZ+ strain (compare Fig. 2B to A and D to C, parent strains). This residual expression was more important in pyruvate than in glucose and was pHo dependent. These data show that in the absence of EnvZ, *ompC* expression is also influenced by both the pHo and the carbon source. So, without EnvZ, OmpR would be phosphorylated by an EnvZ-independent mechanism and OmpR-P would accumulate because of the lack of EnvZ phosphatase activity. *ompC* expression levels indicate that the OmpR-P level would be higher at low than at high pHo and in pyruvate than in glucose. In pyruvate, OmpR phosphorylation would allow OmpR-P levels high enough to repress *ompF* and strongly activate *ompC*. In glucose, compared to pyruvate, OmpR phosphorylation would be weaker and the OmpR-P accumulation would allow *ompF* activation and lower *ompC* activation.

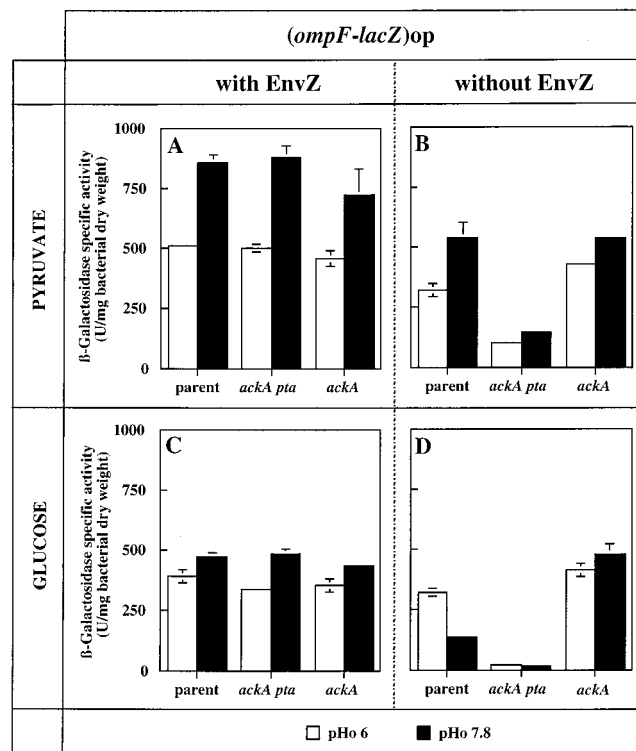From our results collected with and without EnvZ, we can

FIG. 1. Expression of *ompF* at pHos 6 and 7.8 in parental and mutant (*ackA pta* or *ackA*) strains in the presence or absence of EnvZ. Strains were grown in LO medium with pyruvate or glucose as the carbon source. Panels: A and C, GPH8252 parent strain, GPH8255 Δ(*ackA pta*) strain, and GPH8257 *ackA200* strain; B and D, GPH8268 parent strain, GPH8273 Δ(*ackA pta*) strain, and GPH8274 *ackA200* strain. The data are mean values ± standard deviations from three independent experiments.

deduce that both EnvZ activities would not be responsible for the pHo regulation and the carbon source-dependent activation of porin genes. In addition to the kinase activity of EnvZ, OmpR phosphorylation would require pHo- and carbon source-dependent mechanisms.

Role of noncognate histidine kinase in *ompF* pHo regulation. In an EnvZ-deficient strain, *ompF* transcription remained pHo dependent (Fig. 1B and D, parent strains). Since CheA was demonstrated to phosphorylate OmpR in vitro (9), we investigated the physiological relevance of cross-regulation between noncognate kinases (PhoR, CheA, and CreC) and OmpR when EnvZ is absent and the role that this cross-regulation may play in the pHo regulation of *ompF*. The β-galactosidase activities of isogenic *phoR20*, Δ(*cheA-cheZ*), and *creB creC* derivatives of an *envZ22* strain, carrying an *ompF-lacZ* operon fusion, were assayed during growth in LB broth adjusted to different pHs and compared with the activities in the *envZ22* mutant. *ompF* expression was found not to be significantly modified in the presence of these mutations (data not shown). These results indicate that in an EnvZ-deficient strain, in vivo cross-regulation between noncognate histidine kinases (PhoR, CheA, and CreC) and OmpR does not play any physiological role in *ompF* pHo-dependent transcription.

Role of AcP in *ompF* and *ompC* pHo regulation. AcP is synthesized from acetyl coenzyme A and P$_i$ with the release of free coenzyme A by phosphotransacetylase. AcP and ADP are then converted to acetate and ATP by acetate kinase. An *ackA* mutant is expected to accumulate AcP because its breakdown is blocked. An *ackA pta* mutant is expected to display a low

AcP level because its synthesis no longer occurs and the phosphotransacetylase-acetate kinase pathway cannot be inverted (10). Use of pyruvate as a carbon source was described to lead to a fourfold higher AcP level than the use of glucose during the exponential growth phase (12). To determine the role of AcP in OmpR activation, we analyzed the expression of *ompF-lacZ* and *ompC-lacZ* operon fusions with glucose or pyruvate as the carbon source and in the presence of an *ackA pta* or *ackA* mutation. β-Galactosidase activities were assayed during the growth of isogenic strains (Table 1) in LO minimal medium adjusted to pH 6 or 7.8 (Fig. 1A and C and 2A and C).

In the presence of EnvZ, with a low AcP level, and in pyruvate or glucose, *ompC* was not significantly induced at pHo 6, leading to pHo-independent expression (Fig. 2A and C, *ackA pta* strain). This result indicates that in the presence of EnvZ, AcP would contribute to OmpR phosphorylation during growth at pHo 6. Without this contribution, OmpR-P levels would be the same at both pHos. Unexpectedly, the 1.7-fold induction of *ompF* during growth with pyruvate at pHo 7.8 compared to pHo 6 was not significantly modified when the AcP level was decreased (Fig. 1A, *ackA pta* strain). When AcP was high, whatever the carbon source, *ompC* expression was almost the same at both pHos, meaning that OmpR-P levels were the same at both pHos (Fig. 2A and C, *ackA* strain). *ompF* expression was not modified, whatever the pHo, by a high AcP level (Fig. 1A and C, compare parent and *ackA* strains).

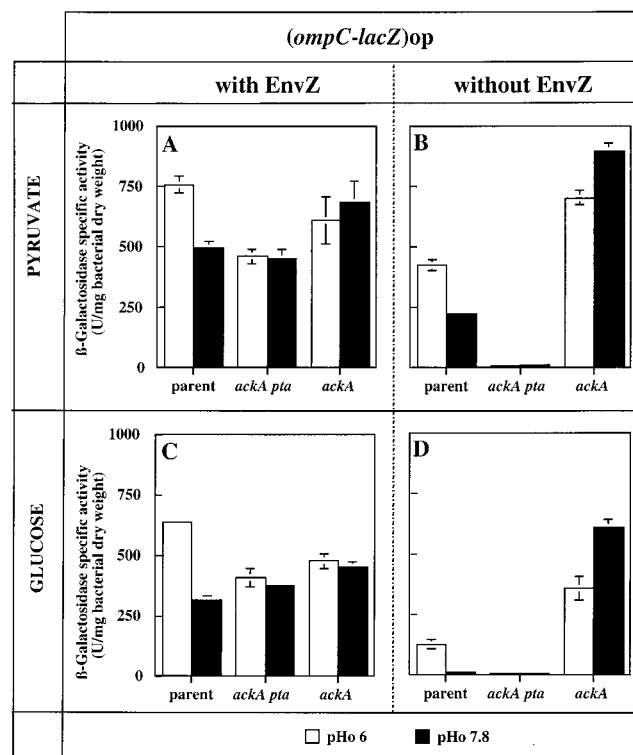We next tested if OmpR activation requires AcP in the

FIG. 2. Expression of *ompC* at pHos 6 and 7.8 in parental and mutant (*ackA pta* or *ackA*) strains in the presence or absence of EnvZ. Strains were grown in LO medium with pyruvate or glucose as the carbon source. Panels: A and C, GPH8259 parent strain, GPH8282 Δ(*ackA pta*) strain, and GPH8280 *ackA200* strain; B and D, GPH8285 parent strain, GPH8293 Δ(*ackA pta*) strain, and GPH8291 *ackA200* strain. The data are mean values ± standard deviations from two to four independent experiments.

absence of EnvZ. We assayed β-galactosidase activities of iso-genic *envZ22*, *envZ22 ackA pta*, and *envZ22 ackA* derivatives carrying an *ompF-lacZ* or *ompC-lacZ* operon fusion (Table 1) during growth in LO medium adjusted to pHo 6 or 7.8 and supplemented with pyruvate or glucose as the carbon source (Fig. 1B and D and 2B and D). In the absence of EnvZ and AcP, *ompF* was no longer expressed in glucose (Fig. 1D, *ackA pta* strain) but was still expressed in pyruvate although at a lower level than in the presence of AcP (Fig. 1B, compare *ackA pta* to parent strains). This expression was slightly pHo depen-dent. These results indicate that in the absence of EnvZ, *ompF* transcription is controlled by an AcP-dependent mechanism. Moreover, *ompF* residual expression during growth in pyruvate evidences apparently pyruvate-dependent activation of *ompF* (Fig. 1B, *ackA pta* strain). This mechanism, independently of EnvZ and AcP, would allow OmpR phosphorylation only dur-ing growth in pyruvate. Pyruvate-dependent activation of the phosphate regulon was previously reported by Wanner and Wilmes-Riesenberg (22). In the absence of the P$_i$ sensor (PhoR) and its homolog CreC, pyruvate led to the activation of alkaline phosphatase synthesis. When the AcP level was high, *ompF* expression increased up to the parental level and be-came pHo independent (Fig. 1B and D, *ackA* strain). In this strain, lacking EnvZ phosphatase activity, the OmpR-P formed by the AcP-dependent mechanism accumulates to a level cor-responding to *ompF* activation (8).

In the absence of EnvZ and AcP, *ompC* was not expressed (Fig. 2B and D, *ackA pta* strain). This result indicates that whatever the carbon source, AcP is responsible for *ompC* ex-pression when EnvZ is absent. We assume that the pyruvate-dependent OmpR phosphorylation evidenced with *ompF* would yield an OmpR-P level too low to activate *ompC*. With an increased AcP level, *ompC* expression was increased, with slightly higher expression at pHo 7.8 than at pHo 6 (Fig. 2B and D, *ackA* strain). These results correlate well with the resistance of the *envZ22 ackA pta* derivative (strain GPH8273) and the sensitivity of the *envZ22 ackA* derivative (strain GPH8274) to bacteriophage MeI, which uses OmpC as a re-ceptor.

Unexpectedly, growth of an EnvZ-deficient strain with pyru-vate and a high level of AcP was associated with high expres-sion of both the *ompF* and *ompC* genes (Fig. 1B and 2B, *ackA* strains). Such a phenotype was previously described by Mc-Cleary and Stock (12) for an *envZ*$^+$ *pta* strain during growth with sodium acetate and by Russo et al. (17) for a mutant with an OmpR protein unable to repress *ompF*.

In the absence of EnvZ, pHo-dependent expression of *ompF* and *ompC* was clearly demonstrated to depend upon the AcP amount, which would be higher at pHo 6 than at pHo 7.8. To obtain direct evidence of pHo modulation of the AcP level, we tried to directly measure AcP by enzymatic methods during growth at different pHos but the concentration of this com-pound in cells proved to be too low under our conditions to be quantitatively assayed.

Conclusion. In the absence of EnvZ, our results show that *ompF* and *ompC* expression is influenced by pHo and AcP. A higher OmpR-P level at low pHo than at high pHo might be responsible for this regulation. We propose that with or with-out EnvZ, the AcP amount is influenced by pHo, leading to modulation of the OmpR-P level and, consequently, pHo-de-pendent expression of *ompF* and *ompC*. According to our results obtained with and without EnvZ, both EnvZ activities are not involved in pHo regulation of the porin regulon. We do not know whether OmpR is phosphorylated directly by AcP or indirectly through a noncognate kinase, as suggested by Kim et al. (10). AcP was also shown to be responsible for *ompF* and

ompC osmoregulation in the absence of EnvZ (11; Kim and Wanner, personal communication). Our study also suggests that the OmpR-P level is higher during growth in pyruvate than during growth in glucose, when EnvZ is absent. Indeed, under our assay conditions, *ompF* and *ompC* expression de-pends on the nature of the carbon source. Similar results were described by Kim et al. (10). The link between the carbon source and the expression of *ompF* and *ompC* might simply be explained by the modulation of the AcP level as a function of the carbon source, this hypothesis cannot account for all of our results. Indeed, *ompF* residual expression, in the absence of AcP and EnvZ, was still higher in pyruvate than in glucose (compare Fig. 1B to D, *ackA pta* strain), so it may be that a specific signal leads to OmpR phosphorylation in response to pyruvate. This pyruvate-dependent signal would not depend on EnvZ and AcP. Pyruvate-dependent activation of the phosphate (22) and porin regulons should involve different mechanisms. Indeed, AcP is required for pyruvate-dependent activation of the phosphate regulon but not for pyruvate-dependent activa-tion of the porin regulon. This regulon constitutes another two-component regulatory system affected by a control linked to the use of pyruvate as a carbon source. This control might be of more general interest.

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