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# Role of the Transcriptional Activator AppY in Regulation of the *cyx appA* Operon of *Escherichia coli* by Anaerobiosis, Phosphate Starvation, and Growth Phase

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**Transcriptional *lacZ* fusions have been used to analyze the regulation of the *appA* operon of *Escherichia coli*. The *appA* operon contains the genes *cyxA* and *cyxB*, coding for the putative third cytochrome oxidase, and *appA*, encoding acid phosphatase. The analysis showed that the *cyxAB* and the *appA* genes are cotranscribed from a potentially strong promoter, $p_{cyx}$, located immediately upstream of *cyxA* and that the operon in addition contains an internal promoter, $p_{appA}$, contributing significantly to the transcription of the *appA* gene. The two promoters were both induced by starvation for $P_i$ and by entry into stationary phase. The *cyx* promoter was in addition found to be activated by anaerobic growth conditions. The product of the previously identified *appY* gene, which when present on a high-copy-number plasmid stimulates synthesis of acid phosphatase, was shown to activate the *cyx* promoter. An insertion mutation in the *appY* gene was constructed in vitro and recombined into the chromosome. The *appY* mutation eliminated induction of the *cyx* promoter by anaerobiosis and severely reduced induction of this promoter by phosphate starvation and upon entry into stationary phase but had no effect on induction of the *appA* promoter. The *appY* mutation had no effect on survival in stationary phase, nor did it have any effect on growth rate or yield under aerobic or anaerobic conditions. The possibility that AppY is a third global regulator of energy metabolism genes is discussed.**

*Escherichia coli* is a facultative anaerobe organism that can obtain energy by substrate level phosphorylation as well as by oxidative phosphorylation driven by the proton motive force generated by the electron transport chain. Under aerobic conditions, *E. coli* synthesizes two different cytochrome oxidases, *o* and *d*, which perform the terminal step in the electron transport chain reducing oxygen to water (see reference 31 for a review). Cytochrome *d* oxidase, encoded by the *cyd* operon, has a 10-fold lower $K_m$ value for oxygen than cytochrome *o* oxidase, encoded by the *cyo* operon (33). Correspondingly, the *cyo* operon is expressed maximally under oxygen-rich conditions and repressed under anaerobic conditions, whereas the *cyd* operon is induced by semianaerobic conditions (10, 17). During anaerobiosis, *E. coli* can obtain energy by substrate level phosphorylation in the presence of a fermentable carbon source or by oxidative phosphorylation when there is an alternative electron acceptor present (see reference 25 for a review).

The *appA* gene coding for acid phosphatase (pH 2.5), AppA (11), is one of the genes induced by anaerobiosis (12). Recently, it was found that the region upstream of the *appA* gene encodes two proteins homologous to the two cytochrome oxidase *d* subunits (13). The very high level of homology strongly suggests that these genes encode a third *E. coli* cytochrome oxidase. We have therefore chosen to denominate these genes *cyxA* and *cyxB* instead of *appC* and *appB*.

The synthesis of AppA is also induced by entry into stationary phase and by starvation for $P_i$ (12). Recently, it was found by Greiner et al. (18) that the natural substrate for AppA probably is phytic acid [*myo*-inositol (1, 2, 3, 4, 5, 6)hexakiphosphate]. The induction by phosphate starvation has been shown to be independent of the *pho* regulon (41). Two promoters for the *appA* operon have been found: one located in front of the *appA* structural gene and one located upstream of the *cyxA* gene (13). The upstream promoter was shown to be induced by anaerobiosis (13).

Two regulators involved in adjusting the transcriptional rate of genes of *E. coli* shifting from aerobic to anaerobic growth have been identified: the *fnr* gene product and the two-component sensor-regulator system ArcB-ArcA. The *fnr* gene encodes the fumarate-nitrate reductase regulator. Under anaerobic conditions, Fnr activates transcription of several anaerobic respiratory genes (for a review, see reference 39) and the *pfl* gene encoding pyruvate-formate lyase (32, 34) and represses transcription of the *cyd* operon (10). During anaerobic growth, ArcA represses transcription of several genes involved in aerobic metabolism, including the *cyo* operon (23), and activates transcription of the *pfl* gene (35) and the *cyd* operon (15).

We have previously isolated a global regulator gene, *appY*, which when present on a high-copy-number plasmid greatly stimulates synthesis of acid phosphatase from the *appA* gene and affects the rate of synthesis of at least 30 other proteins (2). The AppY protein is homologous to the transcriptional activators of the AraC family (16). In the present study, we have constructed transcriptional fusions of the *appA* operon promoters to the *lacZ* and *phoA* indicator genes and used these fusions to study regulation of the *appA* operon by environmental factors and by AppY protein. To assess the role of the AppY protein in regulation of the *appA* operon by the different environmental treatments, we constructed an *appY* mutant in vitro and recombined it into the chromosome. In the accompanying paper (7), we show that AppY also regulates transcription of the *hya* operon, which encodes hydrogenase 1 and is located immediately upstream of the *appA* operon.

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TABLE 1. *E. coli* K-12 strains used in this study

| Strain | Genotype[a] | Source, reference, and/or construct |
|---|---|---|
| TC3264 | *thi-1 leu-6 thr-1 lacY1 lacI-ZΔ (Mlu) supE44 tonA21 rpsL rfbD1* | 32 |
| LJ24 | *thi-1 leu-6 lacY1 lacI-ZΔ (Mlu) supE44 tonA21 rpsL rfbD1* | 32 |
| TC3266 | *fnr-1 zci*::Tn*10*[b] | 32 |
| TC3538 | *purE zbb-2419*::Tn*10*[c] | 7 |
| UT5600 | Δ(*appY-ent*) | 26 |
| TC3594 | Δ(*appY-ent*)[c] | UT5600(P1) × TC3538 |
| TC3572 | *appY*::*aphA-3561*[b] | This work |
| TC3517 | *appY*::*aphA-3561*[c] | TC3572(P1) × LJ24 |
| TC3981 | *attB*::$p_{cyx}$-*lacZ*[c] | This work |
| LB114 | *purE zbb-2419*::Tn*10 attB*::$p_{cyx}$-*lacZ*[c] | TC3538(P1) × TC3981 |
| LB117 | *appY*::*aphA-3561 attB*::$p_{cyx}$-*lacZ*[c] | TC3572(P1) × LB114[d] |
| TC3504 | LJ24/pACYC184 | |
| TC3505 | LJ24/pTAC3411 | |

[a] Genetic symbols are used according to the Bachmann system (4).
[b] Genotype otherwise like that of TC3264.
[c] Genotype otherwise like that of LJ24.
[d] For details, see the construction of strain LB119 (7).

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study are all derivatives of the C600 Δ*lac* strain TC3264 (32) and are listed in Table 1.

**Growth media and culture conditions.** Luria-Bertani (LB) medium (28) was used for all cloning experiments. The indicators 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl phosphate (X-P) were added to plates at concentrations of 40 and 80 μg/ml, respectively. AB minimal medium (9) supplemented with 1 μg of thiamine per ml, 0.2% glucose, and 1% Casamino Acids (Bacto Difco) was used for growth phase experiments. Unless otherwise stated, stationary-phase cultures were obtained by growth overnight in tubes with 10 ml of medium in an Infors shaker. For anaerobic growth experiments, we used AB minimal medium supplemented with 1 μg of thiamine per ml, 0.1% glucose, 100 μg of leucine per ml, and (when required) 100 μg of threonine per ml. Phosphate starvation experiments were carried out in MOPS (morpholinepropanesulfonic acid) minimal medium (30) supplemented as described above. High-phosphate medium contained 1.32 mM $K_2HPO_4$, and phosphate starvation medium contained a 10-fold lower concentration. For overnight incubations, the reference aerobic or high-phosphate cultures contained 0.04% glucose. These carbon-starved cultures generally had a two- to threefold higher specific activity of β-galactosidase than cultures grown exponentially in glucose minimal medium. The antibiotics added were ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 10 μg/ml.

Aerobic cultures were grown with 100 ml of medium in 500-ml flasks or in tubes with 10 ml of medium with vigorous shaking. Anaerobic growth was achieved by bubbling nitrogen into culture flasks (7) or by incubation in screw-cap tubes completely filled with medium and sealed with tape. For all experiments, cell density was monitored by the optical density at 450 nm ($OD_{450}$) with a Zeiss PMQ2 spectrophotometer. When the cell density was above 0.5, the samples were diluted appropriately before measurement.

**Enzyme assays.** β-Galactosidase activity was determined by using toluene-permeabilized cells essentially as described by Miller (28). Cell debris were removed by centrifugation when necessary. Alkaline phosphatase activity was determined by using toluenized cells as described by Boquet et al. (6). Acid phosphatase activity was determined as described previously (2).

**DNA technology.** Plasmid DNA was prepared by the method of Birnboim and Doly (5). Restriction enzymes, T4 DNA ligase, DNA polymerase I Klenow fragment, and phosphorylated 10-bp *Xho*I linker were used as recommended by the suppliers (New England Biolabs).

**Construction of plasmids.** Construction of the promoter fusion and *appY* gene-carrying plasmids is described in the figure legends. Plasmid pUF1 (kindly donated by Ulrik von Freiesleben), which was used as a control, carries the *dnaA* promoters pointing towards *lacZ* and the *bla* promoter transcribing *phoA*. pUF1 was constructed by cloning a 744-bp *Hin*dII fragment from pFH539 (21) into *Sma*I-restricted pCB267 (37).

**Construction of a chromosomal $p_{cyx}$-*lacZ* fusion.** The smallest intact *cyx* promoter fragment is carried by plasmid pTAC3604 (see Fig. 4). Before integration into λ*att*, this $p_{cyx}$ fragment was recloned into pTAC3953, the improved version of the promoter-cloning vector (7), by using *Bst*EII and *Hin*dIII. The promoter *lacZ* fusion was integrated into *attB* exactly as described previously, and the correct integration was verified by Southern blot analysis (3). The integrated *lacZ* fusion was transferred to strain LJ24 by P1 transduction, giving rise to strain TC3981.

**Construction of the *appY*::*aphA* mutant.** The *appY*::*aphA* 4.9-kb *Eco*RI fragment of plasmid pTAC3561 (see Fig. 5) was cloned into the *Eco*RI site of the *rep*(Ts) plasmid pSM491, and the *appY* mutation was transferred into the chromosome of strain LJ24 by the procedure described previously (32). The structure of the *appY* region of the chromosome of the resulting *appY* mutant strain TC3572 was verified by Southern blot analysis as described previously (3), with the *appY*-carrying *Hin*dIII-*Sma*I fragment of pTAC3255 (see Fig. 5) as a probe.

## RESULTS

**Construction of *appA* operon promoter fusions.** To localize the promoter activated by the AppY protein, we constructed a series of promoter fusion plasmids carrying different fragments from the region upstream of the *appA* gene by using the promoter probe vector pCB267 (37), which carries a multiple cloning site between promoterless *phoA* and *lacZ* genes (Fig. 1C). Initially, we cloned different restriction fragments from plasmid pTAC3136 (2) carrying 9 kb of DNA upstream of *appA* into pCB267 and selected LacZ- or PhoA-positive clones, the latter being more sensitive for detection of very weak promoters. Subsequently, deletion derivatives of the initial plasmids were constructed to obtain clones carrying smaller promoter fragments.

Plasmids pTAC3470 and pTAC3431 (Fig. 1B) carry the 2.2-kb *Eco*RV fragment spanning the intergenic region between the *hya* operon and the *cyxAB* genes, which contains the *cyx* promoter (designated p2 by Dassa et al. [13]). The absence of significant *lacZ* expression from pTAC3879, pTAC3492, and pTAC3467, which carry fragments upstream of the *Bgl*II site located immediately in front of *cyxA* (Fig. 1), indicates that all upstream transcription of *appA* originates from the promoter identified by Dassa et al. (13). This is confirmed by the identical indicator gene expression from plasmids carrying smaller $p_{cyx}$ fragments derived from the original *Eco*RV clones (Fig. 1B, compare pTAC3431 and pTAC3494).

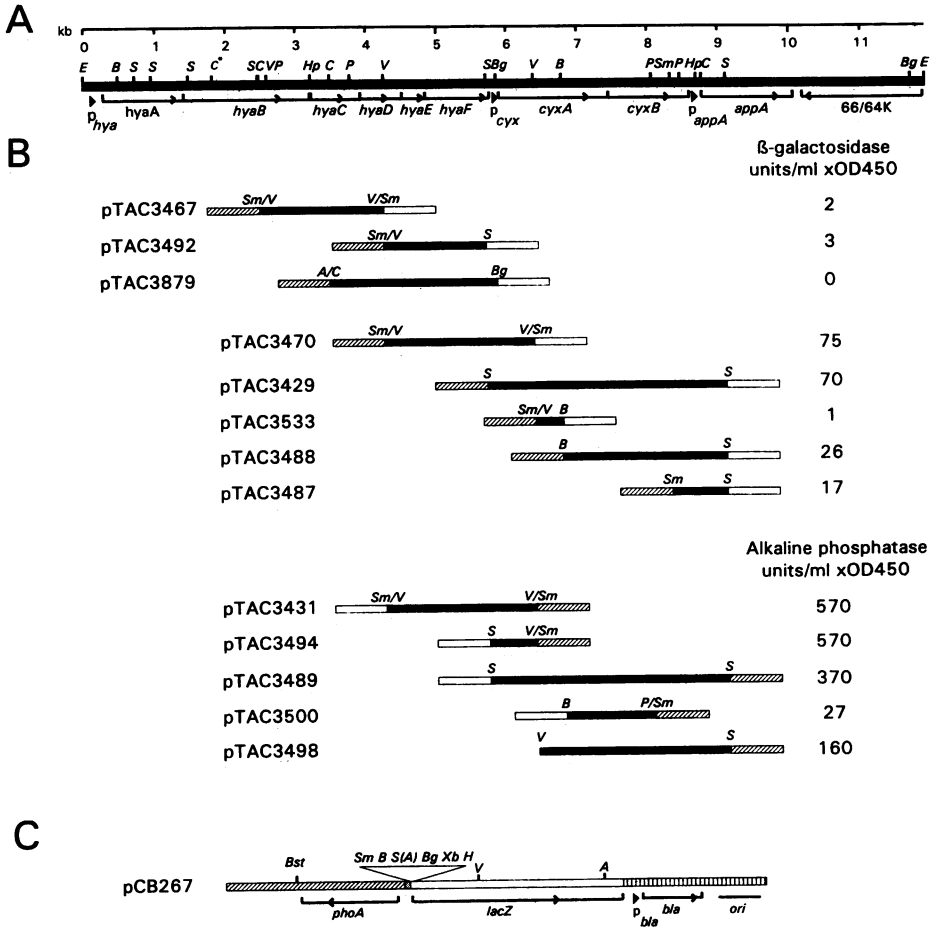


FIG. 1. Transcriptional fusion plasmids for analysis of the *appA* operon. (A) Structure of *Eco*RI fragment carried by plasmid pTAC3136. Isolation of plasmid pTAC3136 was described previously (2). Positions 0 and 10 kb on the scale shown at the top correspond to positions 1046 and 1056 kb, respectively, on the restriction enzyme map of the *E. coli* chromosome (24). Position 0.114 kb corresponds to position 1 in the *hya* sequence (27), position 5.707 corresponds to position 1 in the *appA* operon sequence (13), and position 8.593 kb corresponds to position 1 in the *appA* gene sequence (14). The restriction enzyme sites and the position and extent of the genes are indicated according to the published sequences, except for the nonsequenced region beyond 10 kb. The presence and direction of transcription of the 66/64K protein was deduced from maxicell analysis (1). Two restriction sites (a *Pst*I site at 7.244 kb and an *Hpa*I site at 8.821) present in the published sequences but absent from the DNA carried by pTAC3136 have been omitted. The *Eco*RI site at 7.5 kb in the plasmid of Menon and coworkers is not present in our plasmids, in the DNA sequenced by Dassa et al. (13), or on the Kohara map (24). (B) Structure of the promoter clones derived by cloning fragments from pTAC3136 into the promoter probe vector pCB267. The restriction enzymes used in the cloning are indicated at the junctions of vector and donor DNA for each plasmid. Where two different restriction sites were ligated in the plasmid construction, their symbols are separated by a slash. When necessary, restriction fragments with 5′ overhangs were treated with DNA polymerase I Klenow fragment before ligation to *Sma*I-digested vector. The β-galactosidase or alkaline phosphatase specific activity was determined from duplicate 10-ml cultures of strain LJ24 carrying the plasmid grown to stationary phase in AB minimal medium supplemented with 0.2% glucose and 1% Casamino Acids. All strains were grown in parallel in the same shaker. The specific activity of β-galactosidase or alkaline phosphatase, determined as described in Materials and Methods, is the average for the two cultures after subtraction of the background from strain LJ24 carrying the vector pCB267. (C) Structure of the promoter probe vector pCB267 (37). Symbols: solid bar, chromosomal DNA from the *hya appA* region; open bar, *lacZ* DNA; hatched bar, *phoA* DNA; vertically striped bar, pBR322-derived DNA; dotted bar, synthetic DNA. *A*, *Acc*I; *B*, *Bam*HI; *Bg*, *Bgl*II; *Bs*, *Bst*EII; *C*, *Cla*I; *C**, methylated *Cla*I site; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pvu*II; *S*, *Sal*I; *Sm*, *Sma*I; *V*, *Eco*RV; *Xb*, *Xba*I.

Plasmid pTAC3487 carrying only the promoter region $p_{appA}$ located close to the *appA* gene, designated p1 by Dassa and coworkers (13), was constructed by deleting the *cyx* promoter and most of the *cyxAB* genes from plasmid pTAC3429. No significant promoter activity was detected from plasmids carrying internal fragments from the *cyxAB* genes (pTAC3533 and pTAC3500).

The indicator gene activity of $p_{appA}$ fusions in the stationary-phase cultures grown in the glucose-Casamino Acid-supplemented medium used for the determinations given in Fig. 1 is somewhat lower than that of the $p_{cyx}$ fusions (compare pTAC3487 with pTAC3470 and pTAC3498 with pTAC3431). In stationary-phase cultures in LB medium, the indicator gene activities from the *appA* and *cyx* promoter fusions are very similar (data not shown). The actual values obtained in stationary-phase overnight cultures vary in different experiments (compare data in Fig. 1 and Table 3), probably because of variations in the degree of shaking of the tubes. The two promoters also show very similar activities during exponential growth in glucose-Casamino Acid-supplemented

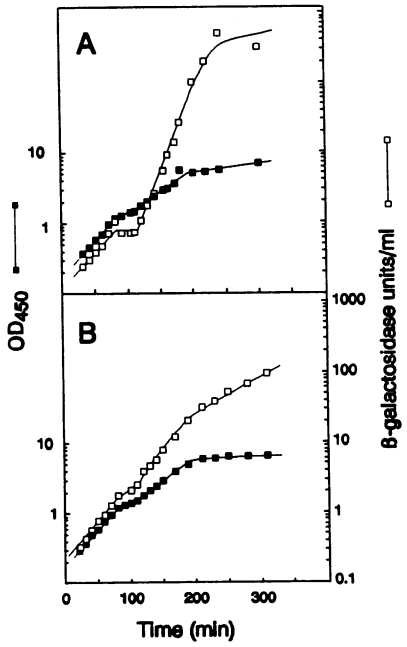


FIG. 2. Growth phase regulation of the *cyx* and *appA* promoters. The LJ24-derived plasmid-carrying strains were grown at 37°C in AB minimal medium supplemented with 0.2% glucose and 1% Casamino Acids. Before the start of the experiment, cultures had been growing exponentially for more than 15 generations. Samples were taken for measurement of $OD_{450}$ and for determination of β-galactosidase activity. (A) $p_{cyx}$-*lacZ* (pTAC3470). (B) $p_{appA}$-*lacZ* (pTAC3487).

medium (Fig. 2) and in minimal glucose medium (Fig. 3C and D).

**Regulation of the *appA* operon promoters by anaerobiosis, growth phase, and phosphate starvation.** Acid phosphatase synthesis is induced by entry into stationary phase (2, 12), by starvation for $P_i$, and by lowered oxygen tension (12). It has been shown that the induction by anaerobiosis is due to increased transcription from the upstream promoter (13). In agreement with this, we found that transcription of the *lacZ* gene in strains carrying $p_{cyx}$ fusion plasmids was strongly stimulated by anaerobic growth conditions (Table 2), whereas *lacZ* expression was unaffected by anaerobic growth conditions in the strain carrying the $p_{appA}$ fusion plasmid pTAC3487 (data not shown).

To identify the promoter(s) responsible for the growth phase regulation, we measured β-galactosidase activity in strains carrying the two promoter *lacZ* fusion plasmids pTAC3470 and pTAC3487 in cultures growing aerobically in phosphate-buffered glucose-Casamino Acid-supplemented medium (Fig. 2). Specific β-galactosidase activity in the strain with the $p_{cyx}$-*lacZ* plasmid (Fig. 2A) increased approximately 100-fold during the deceleration phase of growth, whereas that with the $p_{appA}$-*lacZ* plasmid (Fig. 2B) increased only about 10-fold. Plasmid copy number measured by slot blot (data not shown) increased three to fourfold from exponential growth phase to stationary phase.

With the aim of determining which of the promoters is responsible for the induction of AppA synthesis by starvation for $P_i$, we grew strains carrying the *cyx* and *appA* promoter plasmids in $P_i$-limited MOPS glucose minimal medium (Fig. 3). When the $P_i$ concentration in the medium approached zero, growth became linear with a decreased growth rate and AppA

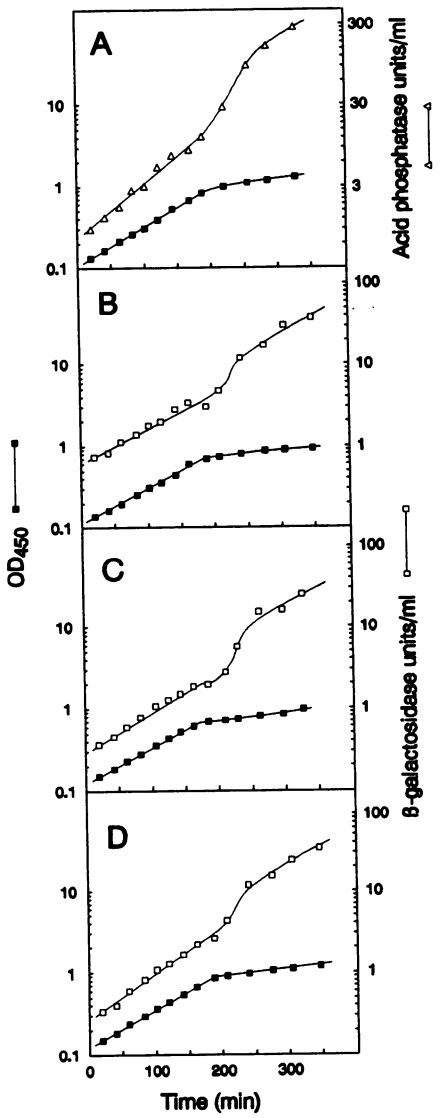


FIG. 3. $P_i$ starvation induction of acid phosphatase and transcription of the *cyx appA* operon *lacZ* fusions. Cultures were grown at 37°C in MOPS minimal medium supplemented with glucose and limiting amounts of $P_i$. Cultures were pregrown exponentially for more than 10 generations before the start of the experiment. Samples were taken for measurement of cell density ($OD_{450}$) and for determination of acid phosphatase activity or β-galactosidase activity. (A) Acid phosphatase determination in control strain (TC3504/pCB267). (B) β-Galactosidase from the $p_{cyx}$ $p_{appA}$-*lacZ* fusion (TC3504/pTAC3429). (C) β-Galactosidase from $p_{cyx}$-*lacZ* fusion (TC3504/pTAC3470). (D) β-Galactosidase from $p_{appA}$-*lacZ* fusion (TC3504/pTAC3487).

synthesis was induced, with the specific activity increasing 20-fold during the 3-h starvation period (Fig. 3A). Transcription from the combined *cyx* and *appA* promoter regions (Fig. 3B) as well as that from the two separate promoters (Fig. 3C and D) was induced upon $P_i$ starvation. In all cases, we found that the specific β-galactosidase activity increased approximately 10-fold during the starvation. Plasmid copy number increased twofold during a 4-h $P_i$ starvation period. The β-galactosidase activity before the onset of starvation was identical to that in high-phosphate medium (data not shown).

To localize the sequences required for induction of the *cyx*

TABLE 2. Localization of sites in the *cyx* promoter region required for AppY stimulation, $P_i$ starvation induction, and anaerobic induction

| Plasmid[a] | β-Galactosidase activity[b] (U/ml × $OD_{450}$) | Fold stimulation of promoter activity[c] by: | | |
|---|---|---|---|---|
| | | High AppY[d] | Anaerobiosis | Low $P_i$[f] |
| pTAC3470 | 7 | 29 | 8 | 5 |
| pTAC3531 | 5.5 | 38 | 10 | 10 |
| pTAC3604 | 3.5 | 68 | 17 | 20 |
| pTAC3491 | —[g] | —[g] | —[g] | —[g] |
| pTAC3490 | 2 | 1 | 2 | 3 |
| pTAC3532 | 6.5 | 1 | 2 | 4 |
| pTAC3605 | 23 | 1 | 2 | 3 |
| pUF1 | 110 | 1 | 2 | 1.5 |

[a] The structure of the plasmids is shown in Fig. 4.
[b] Duplicate cultures of strain LJ24 containing the indicated *lacZ* fusion plasmids were grown with full aeration overnight in AB minimal medium supplemented with 0.04% glucose, and the specific activity of β-galactosidase was determined.
[c] Specific activity of β-galactosidase relative to that in strain LJ24 grown in high-$P_i$ (AB) medium with full aeration.
[d] Cultures of strain TC3505 containing the *lacZ* fusion plasmids grown in AB minimal medium supplemented with 0.04% glucose with full aeration.
[e] Cultures grown anaerobically overnight in AB minimal medium supplemented with 0.1% glucose.
[f] Cultures grown overnight with full aeration in MOPS minimal medium supplemented with 0.1% glucose and limiting $P_i$.
[g] No detectable β-galactosidase activity.

promoter, we made deletion derivatives of the *lacZ* fusion plasmid pTAC3470 (Fig. 4) and measured the β-galactosidase production under different growth conditions (Table 2). The promoter activity from plasmid pTAC3604 was even better regulated than that of pTAC3470, indicating that all sequences required for regulation are present within the 318-bp *Sal*I-*Bst*EII fragment and that a downstream unregulated promoter

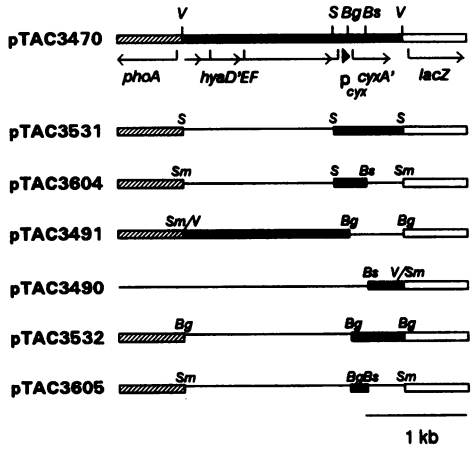


FIG. 4. Structure of plasmids for detailed analysis of the *cyx* promoter region. At the top is plasmid pTAC3470, from which the chromosomal DNA in the other promoter fusion plasmids was derived. Plasmids pTAC3491 and pTAC3490 are *Bgl*II and *Bst*EII deletion derivatives of pTAC3470. Plasmids pTAC3531 and pTAC3532 were constructed by recloning the *Sal*I and *Bgl*II fragments of pTAC3470 into pCB267, taking advantage of the sites in the polylinker. These two plasmids turned out to be somewhat structurally unstable. Therefore, the similar plasmids pTAC3604 and pTAC3605 were made by cloning of Klenow-treated purified fragments into plasmid pTAC3575 (3), a derivative of pCB267 with an *Xho*I linker inserted downstream of *lacZ*. Symbols are defined as in the legend for Fig. 1.

TABLE 3. Effect of AppY overproduction on expression from the *appA* operon promoters[a]

| Plasmid | Promoter(s) | Enzyme measured | Sp act (U/ml × $OD_{450}$) | | Fold stimulation by AppY overproduction[b] |
|---|---|---|---|---|---|
| | | | TC3504 | TC3505 | |
| None | $p_{cyx}$ + $p_{appA}$ | AppA | 600 | 6,700 | 11.0 |
| pTAC3470 | $p_{cyx}$ | β-Gal | 11 | 221 | 20.0 |
| pTAC3429 | $p_{cyx}$ + $p_{appA}$ | β-Gal | 8 | 35 | 4.5 |
| pTAC3487 | $p_{appA}$ | β-Gal | 6 | 8.5 | 1.4 |
| pUF1 | $p_{dnaA}$ | β-Gal | 21 | 25 | 1.2 |

[a] The plasmids carrying the different promoter *lacZ* fusions were transformed into strain TC3504(pACYC184) and strain TC3505(pTAC3411 *appY*$^+$). The specific activity of β-galactosidase (β-Gal) was determined in duplicate stationary-phase cultures grown overnight in AB glucose-Casamino Acid-supplemented medium. The specific activity of acid phosphatase was determined in the strains carrying the control plasmid pUF1. Specific activities are the averages of the two duplicate cultures.
[b] Activity measured in strain TC3505 relative to that in strain TC3504.

(e.g., the one with the low activity detected for pTAC3490) had been deleted. Plasmids pTAC3532 and pTAC3605 (Fig. 4), which carry only the −10 region of the *cyx* promoter (13), showed significant promoter activity. The activity was, however, no longer stimulated by anaerobiosis, strongly suggesting that a sequence required for activation of the promoter is located upstream of or spanning the *Bgl*II site. There was still some induction by phosphate starvation.

**Stimulation of $p_{cyx}$ by AppY protein overproduction.** In order to locate the promoter on which AppY protein overproduction acts to stimulate expression of AppA, the different promoter plasmids were transformed into strain TC3505 carrying the compatible *appY*$^+$ plasmid pTAC3411 and into strain TC3504 carrying the vector pACYC184 and promoter activity was determined in stationary-phase cultures (Table 3). In accordance with previous results (2), the presence of the *appY*$^+$ plasmid stimulated expression of the chromosomal *appA* gene approximately 10-fold. AppY overproduction stimulated expression of the *cyx* promoter 20-fold, whereas $p_{appA}$, like the control promoter $p_{dnaA}$, was virtually unaffected by the presence of the *appY*$^+$ plasmid. Thus, the increased acid phosphatase activity from the chromosome and the increased *lacZ* expression from plasmid pTAC3429, carrying both promoters, must be due to readthrough from the upstream *cyx* promoter. Experiments with the different plasmids carrying $p_{cyx}$ pointing towards *phoA* gave the same results as those with the *lacZ* plasmids (data not shown). As shown in Table 2, the same sequences of the *cyx* promoter are required for stimulation by AppY overproduction and anaerobic induction.

**Construction and phenotypic characterization of an *appY* insertion mutant.** We next wanted to determine the role of the AppY protein in regulation of the *cyx* promoter in an *appY* mutant. A fragment containing the *aphA* gene from Tn*903* was inserted into the *Sca*I site located 34 codons from the C terminus of *appY*. To verify that the insertion inactivated the *appY* gene, we introduced the parental plasmid pTAC3255, the *appY*::*aphA* mutant plasmids pTAC3560 and pTAC3561 (Fig. 5), and the vector pACYC184 into an *appY* deletion strain, TC3594, which contains a 20-kb deletion encompassing the *ompT*, *envY*, and *appY* genes (19), and measured AppA activity. As expected from the previous data on *Bal* 31 deletion mutations (2) that showed that the AppY C terminus is essential for stimulating AppA synthesis, we found that strains carrying plasmids with a mutant *appY* gene had the same level of AppA activity in stationary-phase cultures as the strain with the vector, whereas the strain with the *appY*$^+$ plasmid had a

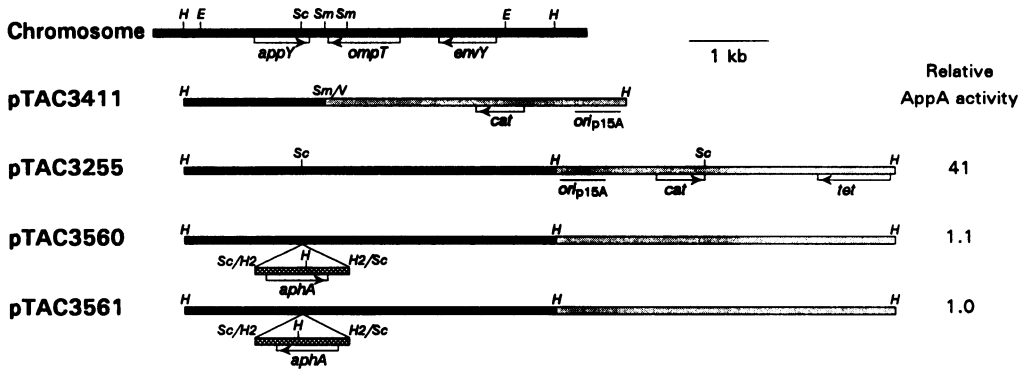


FIG. 5. Structure of the pACYC184-derived *appY*-carrying plasmids. At the top is the structure of the *Hin*dIII fragment carried by the original *appY* isolate plasmid, pTAC3135 (2). All plasmids are derivatives of pACYC184 (8). The restriction enzymes used in the clonings are indicated at the junctions of vector and donor DNAs for each plasmid. Plasmids pTAC3560 and pTAC3561 were obtained by cloning of the 1.2-kb *Hin*cII Tn*903 aphA* fragment derived from pUC4K (40) into plasmid pTAC3255 partially digested with *Sca*I. Plasmids carrying *aphA* inserted into *appY* were isolated by selection for kanamycin and chloramphenicol resistance. The acid phosphatase activity of strain TC3594 carrying the different *appY* plasmids was determined from quadruple overnight cultures in LB medium. Activity relative to strain TC3594 carrying pACYC184 is indicated to the right. Symbols: solid bar, chromosomal DNA from the *appY* region; shaded bar, pACYC184-derived DNA; crosshatched bar, Tn*903*-derived DNA. *E*, *Eco*RI; *H*, *Hin*dIII; *H2*, *Hin*cII; *Sc*, *Sca*I; *Sm*, *Sma*I; *V*, *Eco*RV.

40-fold higher activity (Fig. 5). Additionally, we verified that the level of expression of $p_{cyx}$-*lacZ* fusion plasmid pTAC3604 was completely unaffected by the presence of insertion mutant *appY* plasmid pTAC3561 both in *appY*$^+$ strain LJ24 and in the *appY* deletion mutant strain (data not shown).

The mutant *appY* gene from plasmid pTAC3561 was recloned into a *rep*(Ts) derivative of pSC101 and recombined into the chromosome by the procedure we used previously (32). The presence of the insertion in the *appY* gene on the chromosome of the resulting mutant strain TC3572 was verified by Southern blot analysis (data not shown).

AppY protein affected the synthesis of many proteins in a growth-phase-dependent fashion (2). We therefore compared 3-week survival rates of the wild type (LJ24) and the *appY* mutant (TC3617) strains in stationary-phase cultures at 37°C. Both in LB and in glucose minimal medium, the decay curves of CFU of the two strains were identical (data not shown). As a further confirmation that the *appY* mutant is not affected in development of stationary-phase resistance (22), the stationary-phase cultures of the wild type and the mutant showed identical killing rates upon exposure to $H_2O_2$ and a high temperature (55°C).

Under fully aerobic conditions, *appY* mutant strain TC3617 grows at the same rate as wild-type strain LJ24 (less than 5% reduction) in all media tested, i.e., LB, glucose- and glycerol-Casamino Acid-supplemented media, and glucose and glycerol minimal media. The two strains also have the same growth rate under fully anaerobic growth conditions both in fermentative media, e.g., glucose minimal medium, and during anaerobic respiration in glycerol fumarate medium. Also, the growth yields in glucose minimal medium were identical for the two strains in fully aerobic and fully anaerobic cultures, as well as under semianaerobic growth conditions (i.e., gentle shaking).

**Effect of the *appY* mutation on regulation of the *cyx* promoter by anaerobiosis, growth phase, and phosphate starvation.** In the following experiments, we used a *lacZ* fusion integrated into the λ attachment site to avoid effects from changes in plasmid copy number under the different growth conditions. The $p_{cyx}$-*lacZ* fusion in strains TC3981 (*appY*$^+$) and LB117 (*appY*::*aphA*) corresponds to the promoter fusion plasmid pTAC3604 which carries the smallest and best-regulated *cyx* promoter fragment.

To test for the effect of *fnr* and *appY* mutations on anaerobic induction, the strains were grown exponentially in glucose minimal medium under fully aerobic conditions and parts of the cultures were shifted to anaerobic conditions. In the wild type, we observed a 20-fold induction of the $p_{cyx}$-*lacZ* fusion upon shift to anaerobiosis (Fig. 6). Both the fold induction and the kinetics are very similar to those observed for the plasmid-borne fusion (data not shown). The *fnr* mutation had no effect on expression of the $p_{cyx}$-*lacZ* fusion (Table 4), whereas the anaerobic induction of the *cyx* promoter was completely abolished by the *appY* mutation (Table 4). During aerobic growth in glucose minimal medium, there is a two- to threefold reduction in the specific β-galactosidase activity in the *appY* mutant strain (Table 4 and see Fig. 8), indicating that there is also a small stimulation of the *cyx* promoter by AppY protein under this growth condition.

When the wild-type and *appY* mutant strains carrying the

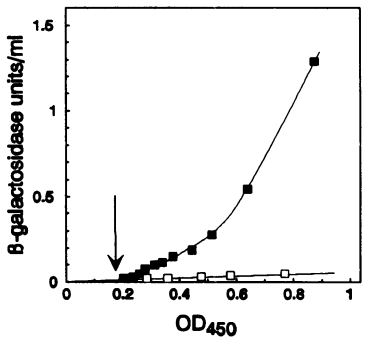


FIG. 6. Effect of shift to anaerobic growth on the differential rate of β-galactosidase synthesis from the $p_{cyx}$-*lacZ* fusion. Strain TC3981 carrying the $p_{cyx}$-*lacZ* fusion integrated into λ*att* was grown aerobically in minimal AB glucose-supplemented medium at 37°C. The culture was pregrown exponentially for more than 10 generations before the start of the experiment. At the point indicated by the arrow, part of the culture was shifted to anaerobic conditions. Samples were taken at 10- to 20-min intervals for measurement of cell density and for determination of β-galactosidase activity. Open squares, aerobic culture; closed squares, anaerobic culture.

TABLE 4. Effect of mutations in the *fnr* and *appY* genes on anaerobic induction of the *cyx* promoter-*lacZ* fusion[a]

| Strain and genotype | Sp act of β-Gal (U/ml × $OD_{450}$) | | Fold induction |
|---|---|---|---|
| | Aerobic | Anaerobic | |
| TC3981 | 0.09 | 1.9 | 21 |
| LB99 (*fnr*) | 0.09 | 1.4 | 16 |
| LB117 (*appY*) | 0.03 | 0.03 | 1 |

[a] The strains were grown exponentially in AB minimal medium supplemented with 0.2% glucose, and six to eight samples were taken over two generations for determination of β-galactosidase activity under aerobic and anaerobic conditions.

chromosomal $p_{cyx}$-*lacZ* fusion were grown exponentially (30-min doubling time) in glucose-Casamino Acid-supplemented medium, transcription from the *cyx* promoter was induced in the wild type in the deceleration phase of growth, with β-galactosidase activity increasing 200-fold (Fig. 7A) similarly to the increase with the plasmid-carried $p_{cyx}$-*lacZ* fusion (Fig. 2). In the *appY* mutant, there was a smaller 15-fold increase (Fig. 7B) upon entry into stationary phase. Increased shaking of the cultures reduced the stationary-phase induction in the wild type to 15-fold (Fig. 1C) and virtually eliminated induction in the *appY* mutant (Fig. 7D), since a twofold increase is expected because of the twofold increase in concentration of the *lacZ* fusion in *att*λ at slow growth rates (20).

The wild-type and *appY* mutant strains carrying the $p_{cyx}$-*lacZ* fusion were grown in phosphate-limited glucose minimal medium to test for the role of AppY protein in induction by phosphate starvation. As shown in Fig. 8, the induction is reduced significantly in the *appY* mutant. The differential rate of β-galactosidase synthesis increased approximately 100-fold in the wild type at the onset of phosphate starvation, whereas it increased only 6-fold in the *appY* mutant.

The *appY* mutation had no effect on growth phase induction or induction by phosphate starvation of the $p_{appA}$-*lacZ* fusion on plasmid pTAC3784 or on induction of the chromosomal *phoA* gene (data not shown). Thus, we conclude that the *appY* mutation specifically reduces the induction of the *cyx* promoter by phosphate starvation and entry into stationary phase.

## DISCUSSION

In this work, we have used *lacZ* fusions to analyze the transcriptional regulation of the *E. coli appA* operon, which contains the genes *cyxA* and *cyxB*, coding for a new cytochrome oxidase, and the *appA* gene, encoding acid phosphatase. Our results show that the three genes are cotranscribed from a potentially strong promoter, $p_{cyx}$, located immediately upstream of *cyxA* and that the operon in addition contains an internal promoter, $p_{appA}$, contributing significantly to the transcription of the *appA* gene. We found that both promoters are induced by starvation for phosphate and by entry into stationary phase, whereas only the *cyx* promoter is activated by anaerobic growth conditions. We have previously identified a regulatory gene, *appY*, which when present on a high-copy-

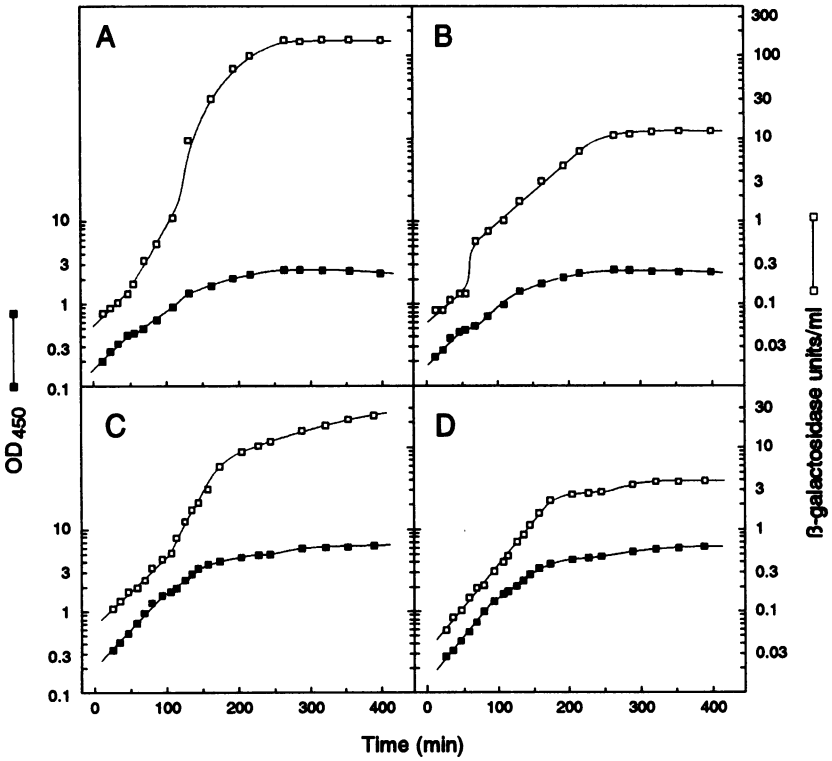


FIG. 7. AppY-dependent growth phase regulation of the *cyx* promoter. Strains TC3981 (*appY*$^+$) and LB117 (*appY*::*aphA3561*) carrying the $p_{cyx}$-*lacZ* fusion integrated into λ*att* were grown at 37°C in AB minimal medium supplemented with 0.2% glucose and 1% Casamino Acids. Before the start of the experiment, cultures had been growing exponentially for more than 15 generations. Samples were taken for measurement of $OD_{450}$ and for determination of β-galactosidase activity. TC3981 and LB117 cultures were grown with rather gentle shaking (80 rpm) (A and B, respectively) or with vigorous shaking (140 rpm) (C and D, respectively).

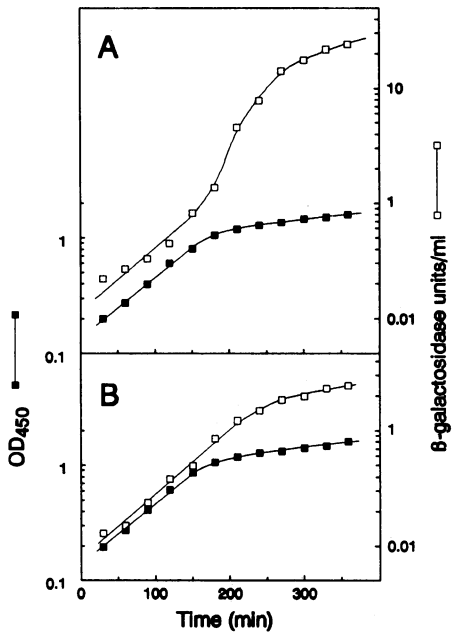


FIG. 8. AppY-dependent $P_i$ starvation induction of the *cyx* promoter. Strains TC3981 (*appY*$^+$) (A) and LB117 (*appY*::*aphA3561*) (B) carrying the $p_{cyx}$-*lacZ* fusion integrated into λ*att* were grown at 37°C in MOPS minimal medium supplemented with glucose and limiting amounts of $P_i$. Cultures were pregrown exponentially for more than 10 generations before the start of the experiment. Samples were taken for measurement of $OD_{450}$ and for determination of β-galactosidase activity.

number plasmid stimulates synthesis of acid phosphatase (2). Here, we show that the AppY protein regulates AppA synthesis by activating transcription from $p_{cyx}$. Using the *appY* mutant constructed in this work, we found that AppY is essential for anaerobic induction of $p_{cyx}$. Induction of the *cyx* promoter by entry into stationary phase and by phosphate starvation is severely reduced in the *appY* mutant, whereas induction of the *appA* promoter is unaffected.

Our genetic analysis of the *cyx* promoter region indicates that the presence of sequences upstream of the −10 region are required for activation of the *cyx* promoter by AppY protein and anaerobiosis, suggesting that the AppY binding site is located in this region.

We found that the *cyx* promoter was regulated similarly by all three inducing conditions whether it was located as a single-copy fusion in λ*att* or was present on a high-copy-number plasmid. This result indicates that the regulatory factors for the *cyx* promoter, and particularly AppY protein, are present in fairly high concentrations in the cell. Alternatively, the *appY* gene might be autoregulated, with the introduction of extra binding sites leading to increased transcription of the *appY* gene.

Expression of acid phosphatase is strongly induced by starvation for $P_i$ (12), and this induction was shown to be independent of the *pho* regulon (42). From the present study, we can conclude that this induction is at the transcriptional level and is mediated by induction of both $p_{cyx}$ and $p_{appA}$. The induction of the *cyx* promoter was found to be reduced in the *appY* mutant, indicating that part of the response is mediated by AppY protein. The residual induction of $p_{cyx}$ in the mutant corresponds to that observed for the deletion derivatives of the *cyx* promoter where sequences upstream of position −20 were replaced by vector sequences.

So far, no regulatory factor for the *appA* promoter has been identified. Since transcription is induced to the same degree by phosphate starvation and upon entry into stationary phase in high-phosphate medium, it might be that this promoter is responding to growth rate, which decreases both upon phosphate starvation and during the deceleration phase of growth.

The induction of the *cyx* promoter upon entry into stationary phase is much stronger than that of the *appA* promoter and is strongly dependent upon AppY protein. We think that it is very likely that during the deceleration phase of growth the AppY protein is responding to the same signal as that elicited by anaerobiosis. The signal is probably not the oxygen or redox state, since the AppY-dependent growth phase induction also took place in well-aerated cultures, but could be a change in the concentration of a metabolite interacting with the AppY protein.

The kinetics of the induction of the *cyx* promoter upon shift to anaerobic conditions is quite different from that observed for most genes. The $p_{cyx}$ induction becomes maximal only about one mass doubling after the shift to anaerobiosis, whereas maximal induction of the *pfl* promoter and of the synthesis of many other proteins occurs immediately (32, 38). Since transcription of the *appY* gene is induced under anaerobic conditions (1), this could be due to increased synthesis of AppY protein at the onset of anaerobiosis, and thus only as the concentration of AppY builds up does the *cyx* promoter become fully activated.

Although we have previously observed that overproduction of AppY protein affects the synthesis of many proteins in a growth-phase-dependent way, we did not find any defects in the *appY* mutant in survival or development of stress protection in stationary phase. Nor did we find any effect on growth rate or growth yields. In the accompanying paper (7), we show that the AppY protein is also involved in anaerobic induction of transcription of the *hya* operon. The *hya* operon is located immediately upstream of the *appA* operon and encodes hydrogenase 1, which probably functions in the energy-conserving hydrogen uptake process (36). We therefore propose that AppY could be a third global regulator for energy metabolism genes in addition to the two previously identified regulators, Fnr and the ArcA-ArcB system.

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