JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1683–1691
0021-9193/89/031683-09$02.00/0

Vol. 171, No. 3

Isolation, Characterization, and Nucleotide Sequence of *appY*, a Regulatory Gene for Growth-Phase-Dependent Gene Expression in *Escherichia coli*

TOVE ATLUNG,* ANNE NIELSEN, AND FLEMMING G. HANSEN

Department of Microbiology, Building 221, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 11 July 1988/Accepted 2 December 1988

A plasmid carrying a regulator gene, designated *appY*, was found in the screening of an *Escherichia coli* gene library for clones overproducing AppA, an acid phosphatase which is induced as a culture approaches the stationary phase. In cells containing multicopy plasmids carrying the *appY* gene, the expression of the chromosomal *appY* gene was stimulated 10- to 40-fold in the stationary phase and more than 100-fold during exponential growth. The *appA* plasmid also changed the rate of synthesis of more than 30 other proteins in a growth-phase-dependent way. The *appY* gene was mapped to 13 min on the *E. coli* genetic map. The position of the *appY* gene on the 4.9-kilobase *Hin*dIII fragment of the original clone was located by Tn*5* mutagenesis and deletion analysis, and the nucleotide sequence of a 1.9-kilobase region containing the gene was determined. The *appY* gene product was identified as a weakly expressed 243-amino-acid polypeptide which contains a stretch of 20 amino acids showing very good similarity to the conserved DNA-binding domain of repressors and transcriptional activators.

Growth-phase-regulated gene expression in the gram-positive bacterium *Bacillus subtilis* has been extensively studied, but so far, little attention has been given to this phenomenon in gram-negative bacteria like *Escherichia coli*. In *B. subtilis*, expression of genes directly involved in sporulation, of exoenzyme genes (29), and of some genes in intermediary metabolism (12) is induced upon entry into the stationary phase. Several genes, *spo0* loci, and genes for alternative sigma factors that are involved in this transcriptional switch have been identified (29).

In members of the family *Enterobacteriaceae*, only few genes responding in a similar manner to growth phase have been found (6, 11, 17, 22, 23, 30, 43; M. Givskov and S. Molin, unpublished results). The genes that have been studied in some detail seem to fall into two regulatory groups, one consisting of genes that require the OmpR protein for expression, and one consisting of genes that belong to the fermentative anaerobic stimulon. The transcriptional activator protein OmpR, best known for its role in osmoregulation of the major outer membrane proteins (21, 32) is required for the expression of the plasmid-encoded *E. coli* microcin B17 (*mbc*) genes (22), the *Salmonella typhimurium tppB* gene coding for a tripeptide permease (18), and the *Serratia liquefaciens* exoenzyme phospolipase, *phlA* (Givskov and Molin, unpublished). The OmpR protein shows extensive homology with the *spo0A* and *spo0F* gene products of *B. subtilis* (15), which are required for transcription of many of the *spo* genes and of the exoenzyme genes (29).

The only other growth-phase-regulated genes in members of the family *Enterobacteriaceae* that have been well characterized are the *pepT* gene of *S. typhimurium* (43) involved in transport of tripeptides; the *E. coli pepN* (17) gene, which codes for aminopeptidase N; and the *appA* gene (11), coding for an acid phosphatase. These three genes, as well as the *tppB* gene, are induced by anaerobiosis; only for *pepT* is the anaerobic induction dependent on the Fnr protein (17, 24, 43; T. Atlung, unpublished results), which is the transcriptional activator for genes involved in anaerobic respiration like the nitrate and fumarate reductase genes (41). The *pepN* and *appA* genes are also induced by starvation for P_i, but the induction is independent of the *pho* regulon, i.e., does not require the *phoR*, *phoB*, or *phoM* gene products (17, 45).

As one approach to the study of growth phase regulation in *E. coli*, we cloned the *appA* gene. In screening for the *appA* clones, we also found a plasmid with a regulatory gene that stimulated expression of the chromosomal *appA* gene. In this paper we describe the isolation and mapping of this gene, which we designate *appY*, and the identification of its gene product. We also report the nucleotide sequence of the *appY* gene and the preliminary characterization of its effect on expression of the *appA* gene and on other *E. coli* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Bacterial strains used in this study are listed in Table 1. The positive selection vector used for construction of the gene libraries was pUN121 (34). Plasmid pACYC184 (8) was used for recloning of the fragments for integration in the *polA*(Ts) strain K549 (37), since this strain was found to be transformed very poorly by pUN121-derived plasmids. Bacteriophages M13mp18 and M13mp19 (47) were used to make the clones for DNA nucleotide sequencing reactions. Phage λ *c*I857 *rex*::Tn*5* *O*am29 *P*am80 *b*221 was used as the Tn*5* donor as described previously (38), and phage P1 *vir* was used for P1 transductions (31).

Growth media. LB medium (31) was used in all cloning experiments including screenings for acid phosphatase production. For maxicell experiments and most growth phase regulation experiments, we used AB minimal medium (10) supplemented with 1 μg of thiamine per ml, 0.2% glucose or glycerol, and 1% Casamino Acids (Difco Laboratories) or a synthetic amino acid mixture lacking methionine (FN19) at the concentrations suggested by Neidhardt et al. (33). Antibiotics were used at the following concentrations: tetracy-

* Corresponding author.

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

Strain	Parent strain	Genotype[a]	Source or reference
CSH69 HfrKL99		*thi relA*	31
JM101 F^+		*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*ZΔM15*)	47
MC1000		*thi* Δ(*ara-leu*)*7679 araD139 lacΔX74 galU galK rpsL*	7
MT102	MC1000	*hsdRK12*	M. Trier Hansen
CSR603		*thi leuB6 proA2 argE3 thr-1 phr-1 recA1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl rpsL31 tsx-33 supE44*	39
K549 Hfr		*thyA rha lac rpsL polA12*(Ts)	37
CSH57		*thi purE leu trp his argG ilv metA* (or *metB*) *rpsL ara lacY gal malA xyl mtl*	31
TC3274	CSH57	Rif^r	Spontaneous mutant
NF1805 Hfr		*thi recA1 srl*::Tn*10*	N. Fiil
NF547		*thi his trp cysC lacΔX74 rpsL relA1*	N. Fiil
C600		*thi-1 thr-1 leu-6 lacY1 supE44 tonA21*	2
TC3253	NF547	*srl*::Tn*10*	P1 (NF1805) × NF547
TC3259	C600	*srl*::Tn*10 cysC*	P1 (TC3253) × C600
TC3264	C600	*lacIZ*Δ(Mlu)	C600[b]
TC3395	TC3264	pUN121	This work
TC3396	TC3264	pTAC3303	This work

[a] Genetic symptoms are those of Bachmann (3).
[b] A deletion from the *Mlu*I site in *lacI* to the *Mlu*I site in *lacZ* was crossed into the chromosome of C600 (to be published elsewhere).

cline, 4 μg/ml; ampicillin, 200 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; rifampin, 100 μg/ml.

Acid phosphatase determination. Colonies carrying recombinant plasmids were screened for increased levels of acid phosphatase (AppA) essentially as described previously (5). This was done as follows. *p*-Nitro-phenylphosphate (3 ml) in 1.5 M formic acid was poured onto an LB plate containing fully grown colonies and incubated for 3 min at room temperature. The reaction mixture was removed, and 1 ml of 10 N NaOH was added to the plate. Colonies overproducing AppA appeared bright yellow. Quantitative measurements were carried out by a modification of the assay described by Touati et al. (44). The activity in stationary-phase cells from overnight cultures was determined by using 1 to 50 μl of culture. For exponentially growing cultures, the cells had to be concentrated by centrifugation. Assays were performed with 0.5 ml of 25 mM *p*-nitrophenylphosphate in 250 mM glycine hydrochloride (pH 2.5) and were terminated by the addition of 1 ml of 1 N NaOH after 5 to 20 min of incubation at 37°C. The A_{410} was determined. One unit was defined as 1,000 × A_{410} per minute per milliliter in analogy with β-galactosidase units (31).

Determination of chromosomal map location of the plasmid insert by gradient of marker transfer. Plasmid pTAC3255 was integrated into the chromosome of the *polA*(Ts) Hfr strain K549 by homologous recombination, by plating plasmid-carrying cells on selective plates (LB plus chloramphenicol) at 42°C. We used the integrants as donors in matings with the multiply marked recipient TC3274 by mixing 0.2 ml of overnight cultures of each strain in 10 ml of LB medium. After 4 h of incubation, the mixture was plated on selective media for selection of either Cam^r or *purE*$^+$ exconjugants. *purE* is the first marker of the recipient transferred by K549. The fraction of recombinants having received the other markers was determined by replica plating and plotted against the map position in a semilog plot (31). Despite the problems in frequencies that could be expected as a result of crossing out of the integrated plasmid upon entry into the *polA*$^+$ recipient, this method gave quite a precise map location, i.e., within less than 2 min of the actual position. The K549 strain can probably be used for nearly any plasmid since it is an efficient donor and transfers more than 80% of the chromosome at a reasonable frequency.

DNA technology. Plasmid DNA was prepared by the method of Birnboim and Doly (4). Restriction enzymes, T4 DNA ligase, BAL 31 exonuclease, DNA polymerase I (Klenow fragment) and phosphorylated 8-base-pair (bp) *Hin*dIII linker were used as recommended by the suppliers (New England BioLabs, Inc.; Boehringer Mannheim Biochemicals; and Amersham International). The nucleotide sequence was determined by the dideoxy-chain termination method, with recombinant M13mp18 or mp19 phages, [^{35}S]dATP, and the universal −40 sequencing primer (New England BioLabs) essentially as described by Sanger et al. (40).

Maxicells and in vivo protein labeling. Plasmid-encoded proteins were detected by using the maxicell method (39). The maxicells were irradiated for 10 s at a distance of 60 cm from a 15 W germicidal lamp, and cycloserine was added to 200 μg/ml 1 h after irradiation. The next day, the cells were labeled with 10 μCi of [^{35}S]methionine for 30 min at 37°C. For determination of patterns of protein synthesis, cultures were grown at 37°C in AB minimal medium supplemented with FN19 amino acids mixture, and cells corresponding to 1 ml of A_{450} = 1 were labeled with 15 μCi of [^{35}S]methionine for 2 min. The cells were harvested, parts of the samples were fractionated into periplasm, and membranes (35) or periplasmic proteins were extracted with $CHCl_3$ (1). Proteins from these fractions, together with untreated samples, were separated by sodium dodecyl sulfate-gel electrophoresis by the method of Laemmli (28) and visualized by autoradiography on Kodak X-ray films.

RESULTS

Cloning of the *appA* and *appY* genes. Four libraries of DNA from *E. coli* CSH69 were constructed by ligation of *Hin*dIII, *Eco*RI, *Bgl*II, and *Bam*HI chromosomal DNA digests into the positive selection vector pUN121, in which inserts in the cloning sites in the *c*I gene lead to expression of the *tet* gene from λpR (34). Tetracycline-resistant transformants of strain MT102 (*appA*$^+$) were screened by plate assay for increased

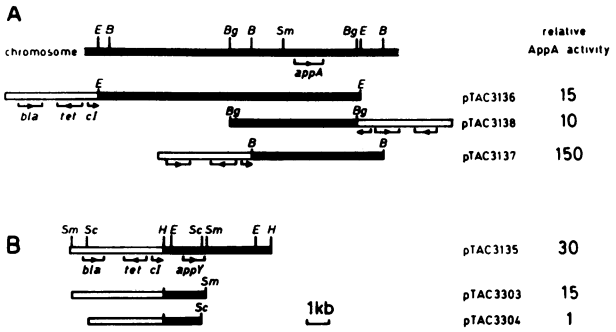

FIG. 1. Structure of plasmids from acid phosphatase-overproducing strains. (A) *appA*-carrying plasmids. At the top is the restriction enzyme map of the *appA* region of the chromosome as deduced from analysis of the plasmids. The map is in complete agreement with that of Boquet et al. (5) and with that of the *E. coli* chromosome at positions 1046 to 1058 (26). (B) The *appY*-carrying plasmid pTAC3135 and two restriction enzyme generated deletion derivatives. Abbreviations: *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *Sc*, *Sca*I; *Sm*, *Sma*I. Symbols: ■, chromosomal DNA; □, vector pUN121 DNA. The extent and position of genes are indicated by brackets and identified by the genetic symbol. The arrows indicate the direction of transcription. The position of the *appA* gene is derived from Boquet et al. (5). To the right is indicated the level of AppA activity in LB stationary-phase cultures of strain MT102 carrying the plasmids. The activity is given relative to MT102 carrying the vector pUN121.

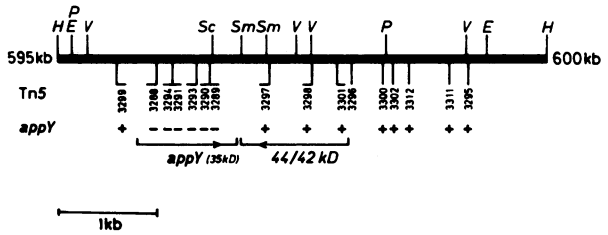

FIG. 2. Tn*5* insertion analysis of plasmid pTAC3135. At the top is the restriction enzyme map of the chromosomal DNA insert in pTAC3135. The positions of the Tn*5* insertions were determined by using the restriction enzyme sites indicated. The horizontal line on the Tn*5* insertion marker indicates the direction of transcription of the *aphA* gene on Tn*5*. Abbreviations: *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *Sc*, *Sca*I; *Sm*, *Sma*I; *V*, *Eco*RV. The *appY* phenotype of the Tn*5* insertion plasmids is given below the insertion numbers. The position and the extent of the two genes indicated at the bottom were deduced from the maxicell analysis in Table 2.

levels of acid phosphatase. One positive clone from each library was chosen for further study.

In overnight cultures, the four clones showed a 15- to 150-fold increase in acid phosphatase (AppA) activity relative to the parental strain (Fig. 1). Restriction enzyme analysis of the recombinant plasmids showed that the *Eco*RI, *Bam*HI, and *Bgl*II clones (pTAC3136, pTAC3137, and pTAC3138, respectively [Fig. 1A]) all contained a common segment of DNA carrying the *appA* gene as deduced from comparison with the restriction enzyme map of Boquet et al. (5).

The *Hin*dIII clone, pTAC3135, however, carried a fragment with a completely different restriction enzyme map (Fig. 1B). Since *E. coli* contains several acid phosphatases with different pH optima (45) in addition to that encoded by *appA*, which has an optimum at pH 2.5, we analyzed the pH optima of the enzyme activity from crude extracts of the strains carrying the four plasmids and from the parental strain. The acid phosphatase in the four recombinants all had exactly the same optimum, and we therefore concluded that the increase in enzyme activity in strain TC3135 was due to increased expression of the chromosomal *appA* gene.

Chromosomal map location of the insert in pTAC3135. To find whether the insert in pTAC3135 carried the *appR* gene, a locus at 58.7 min between *srl* and *cysC* (44) which affects the level of *appA* activity, we mapped the position of the insert on the chromosome. Plasmid pTAC3255, a pACYC184 derivative carrying the *Hin*dIII fragment of pTAC3135, was introduced into the *polA*(Ts) Hfr strain K549, and the plasmid was integrated into the chromosome by selection for Cam^r at 42°C. The vector itself gave no Cam^r colonies at 42°C, indicating that plasmid pTAC3255 integrated by homologous recombination with the chromosome. In P1 transductions with strain TC3259 (*cysC srl*::Tn*10*) as the recipient and four independent recombinants as donors, no cotransduction of Cam^r and *cysC*$^+$ or *srl*$^+$ was observed.

The position of the integrated plasmid was then determined in these four recombinants by using a gradient of marker transfer with TC3274, a Rif^r derivative of the multiply marked strain CSH57, as the recipient. This experiment suggested that the plasmid in all four isolates was integrated close to *purE* at 12.2 min. Subsequent P1 transductions with one of the integrants as donor and the CSH57 strain as recipient showed that the integrated plasmid was 38 or 77% cotransducible with *purE* selecting Cam^r or PurE$^+$, respectively.

Finally, a comparison of the restriction enzyme map of pTAC3135 with that of the *E. coli* chromosome (26) showed that the 4.9-kilobase (kb) *Hin*dIII fragment carried by pTAC3135 is located at 595 to 600 kb, i.e., just clockwise of *purE* at 13.0 min on the standard *E. coli* chromosome map (3). We have designated the gene at this location *appY*.

Localization of the *appY* gene on plasmid pTAC3135. Two deletion derivatives of pTAC3135, made by using available restriction enzyme sites (Fig. 1B), located the *appY* gene to the left 1.9-kb region of the insert. A more precise localization of the gene was attempted by Tn*5* insertion mutagenesis of plasmid pTAC3135. Approximately 30 independent insertions were tested for the AppY phenotype, and the position and orientation of the Tn*5* insertion was determined by restriction enzyme analysis (Fig. 2; only insertions in the chromosomal DNA carried on the plasmid are shown).

The Tn*5* analysis defined the size of the *appY* gene to a minimum of 500 bp and a maximum of 1,300 bp. Two series of BAL 31 deletions were made from either end of the insert in plasmid pTAC3303. *Hin*dIII linkers were inserted to facilitate mapping of the extent of the deletions (Fig. 3). This analysis showed that one end of the *appY* gene is located between 70 and 230 bp from the *Sma*I site at one limit of the insert in pTAC3303, since plasmids pTAC3380 and pTAC3381 are *appY*$^+$, whereas plasmid pTAC3385 is *appY*. Some of the BAL 31 deletions were subsequently recloned into M13 for DNA sequence analysis (see Fig. 4 and 5).

Analysis of gene products from the *appY* region. Maxicell analysis of plasmid-encoded gene products was carried out by using the plasmid pTAC3135, its deletion derivatives shown in Fig. 1B, and a selection of the *Tn5* insertion derivatives (Fig. 2); the results are summarized in Table 2.

Three polypeptides of molecular mass 44, 42, and 35 kilodaltons (kDa) were synthesized from the 4.9-kb *Hin*dIII insert in plasmids pTAC3135, pTAC3255, and pTAC3157, respectively (the last two are pACYC184 derivatives carrying the 4.9-kb fragment in opposite orientations). The 44- and

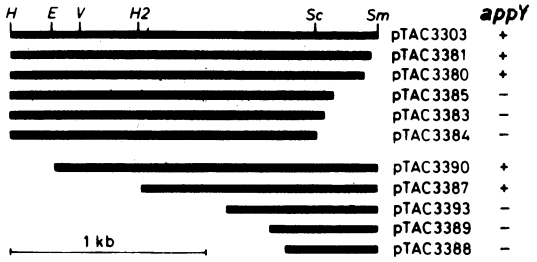

FIG. 3. BAL 31 deletion analysis of plasmid pTAC3303. The first series of plasmids was generated from the *Sma*I site in plasmid pTAC3303 (the complete structure is shown in Fig. 1), and the second was generated from the *Hin*dIII site. Only plasmids having *Hin*dIII linkers inserted were analyzed, and only the chromosomal DNA of the plasmids is shown. The extent of the deletions was determined by restriction enzyme analysis by using the sites indicated above pTAC3303. Abbreviations: *E*, *Eco*RI, *H*, *Hin*dIII; *H2*, *Hin*cII; *Sc*, *Sca*I; *Sm*, *Sma*I; *V*, *Eco*RV.

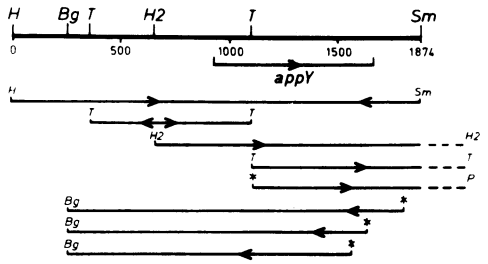

FIG. 4. Strategy for determination of the nucleotide sequence of the 1.9-kb *Hin*dIII-*Sma*I fragment of pTAC3303. The fragments were cloned into M13mp18 or M13mp19 or both by using the indicated restriction enzymes. The arrows indicate the direction and extent of sequence read from each clone. Symbols: ★, BAL 31 deletion, cloned by using the *Hin*dIII linker; *Bg*, *Bgl*II; *H*, *Hin*dIII; *H2*, *Hin*cII; *P*, *Pst*I; *Sm*, *Sma*I; *T*, *Taq*I.

42-kDa products were strongly expressed, whereas the 35-kDa polypeptide was very weakly expressed.

The 44- and 42-kDa polypeptides are probably products of the same gene, the 42-kDa polypeptide being a processed form of the 44-kDa polypeptide, since both were absent from the Tn*5* insertion plasmid pTAC3297 but were made from all the other Tn*5* derivatives tested and since the 44-kDa polypeptide is missing in a sample labeled for 30 min and then chased for 30 min. This hypothesis also fits with the appearance of a corresponding doublet truncated product of 34 and 32 kDa from pTAC3397. The amount of the 44- and 42-kD polypeptides was reduced in maxicells of the Tn*5* insertion plasmid pTAC3396. From these observations, we conclude that the gene is located as shown in Fig. 2 and is transcribed in the counterclockwise direction.

The 35-kDa polypeptide was expressed from all the plasmids that were AppY$^+$ and was missing from all AppY$^-$ plasmids, indicating that it is the product of the *appY* gene. The *Sca*I deletion plasmid pTAC3404 produced a 23-kDa polypeptide, probably a truncated *appY* product. This suggested that the *appY* gene is read in the clockwise direction (Fig. 2).

DNA sequence of the *appY* gene. The nucleotide sequence of the 1.9-kb *Hin*dIII-*Sma*I fragment containing the *appY* gene was determined by the dideoxy-chain termination sequencing method (40). The M13 clones carrying the fragments shown in Fig. 4 were used for the sequencing reactions.

Analysis of the sequence showed the presence of an open reading frame of 243 codons in the clockwise orientation, starting at position 930 (Fig. 5). This open reading frame is preceded by a probable Shine-Dalgarno sequence. No other open reading frames of more than 60 codons were found in either direction. The 243-amino-acid coding sequence is contained within the region defined as carrying the *appY* gene from the Tn*5* insertion and the deletion analysis. The molecular mass of the deduced 243-amino-acid polypeptide is 28,013 Da, whereas the size of the gene product observed in the maxicell analysis was estimated to be 35,000 Da. It is therefore very likely that the ATG at position 930 is the start codon, since the next possible start codon is located 58 codons farther downstream. The size of the truncated product made from the *Sca*I deletion plasmid pTAC3304, which is 221 amino acids as deduced from the DNA sequences, migrates as a polypeptide of 23,000 Da, suggesting that the amino acid sequences responsible for the low migration rate

TABLE 2. Maxicell analysis of *appY* plasmids[a]

Plasmid	Mutation	AppY phenotype	Gene products					
			44/42 kDa	35 kDa	*tet* (42 kDa)	*bla* (30/28 kDa)	*cat* (25 kDa)	Truncated products (kDa)
pTAC3135	None	+	+	+	+	+		
pTAC3304	ΔSca	−	−	−	+	−		23
pTAC3303	ΔSma	+	−	+	+	+		
pTAC3299	Tn*5*	+	+	+	+	+		
pTAC3288	Tn*5*	−	+	−	+	+		
pTAC3291	Tn*5*	−	+	−	+	+		
pTAC3293	Tn*5*	−	+	−	+	+		
pTAC3289	Tn*5*	−	+	−	+	+		
pTAC3297	Tn*5*	+	−	+	+	+		34/32
pTAC3296	Tn*5*	+	(+)[b]	+	+	+		
pTAC3295	Tn*5*	+	(+)	+	+	+		
pACYC184		−	−	−	+		+	
pTAC3255[c]		+	+	+	−		+	
pTAC3257[c]		+	+	+	−		+	

[a] The plasmids were introduced into strain CSR603, and maxicell experiments were carried out, labeling the cells with [^{35}S]methionine. The polypeptides were separated by sodium dodecyl sulfate-gel electrophoresis, and the labeled plasmid-encoded gene products were visualized by autoradiography. A number of Tn*5*-encoded products (not shown in the table) were observed from plasmids with insertions. See Fig. 1 and 2 for structures of the plasmids.

[b] (+), Weakly expressed relative to pTAC3135.

[c] pTAC3255 and pTAC3257 are pACYC184 derivatives carrying the intact 4.9-kb *Hin*dIII fragment in opposite orientations.

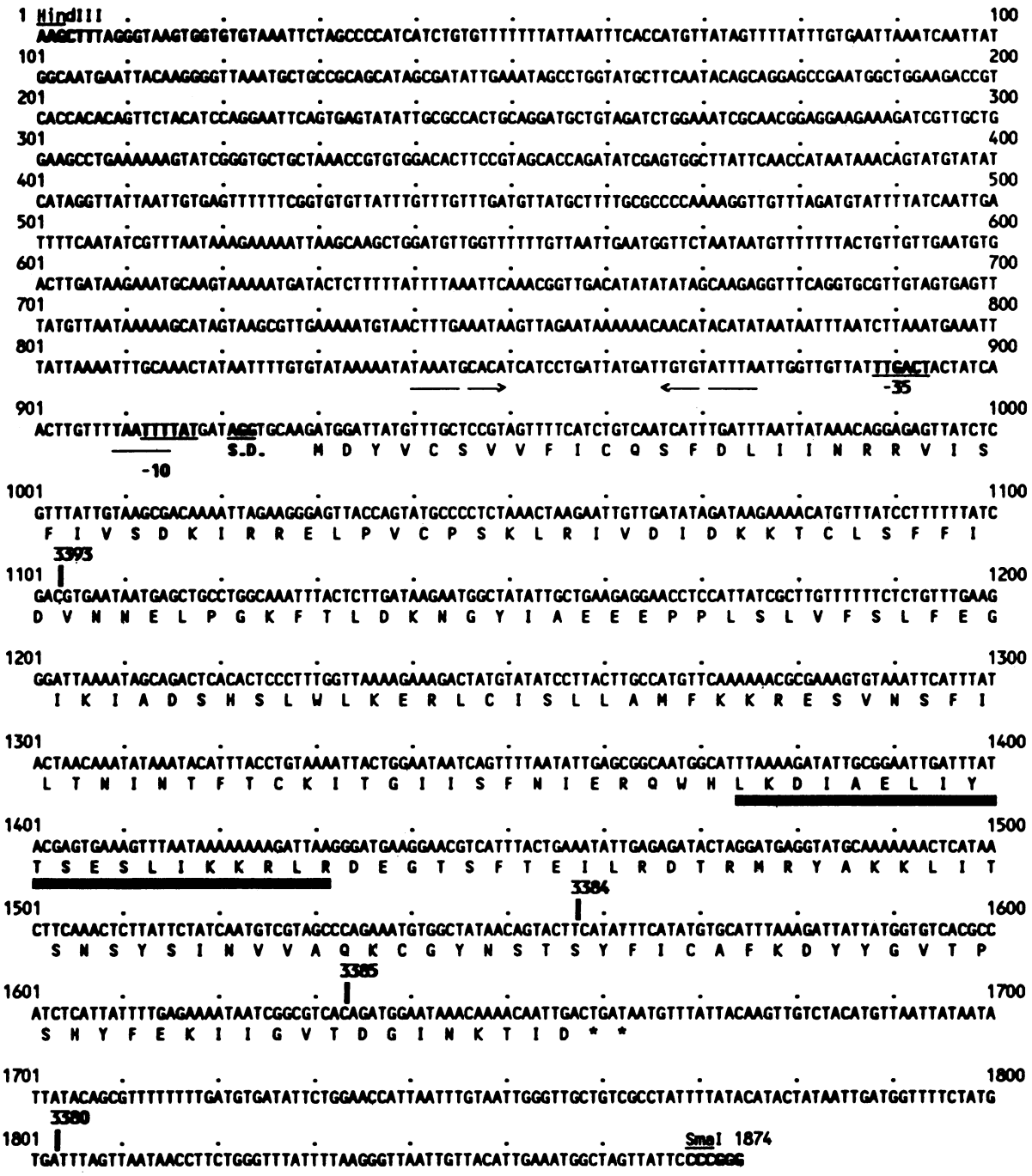

FIG. 5. Nucleotide sequence of the *Hin*dIII-*Sma*I fragment and deduced amino acid sequence of the *appY* gene product. The sequence is numbered from the *Hin*dIII site corresponding to position 595 on the *E. coli* restriction enzyme map (26). The endpoints of BAL 31 deletions (identified by their plasmid numbers from Fig. 3) used in determination of the sequence are indicated above the sequence. The ribosome-binding site preceding the deduced start codon of the *appY* reading frame is indicated by SD. The −35 and −10 sequences of the possible promoter are underlined, and the inverted repeat is indicated by arrows. The probable DNA-binding domain of the protein is indicated by the bold underline.

of the intact product are located in the carboxy-terminal part.

Physiology of strains carrying *appY*$^+$ plasmids. The presence of the *appY*$^+$ plasmid pTAC3303 had no effect on the growth rate of the strain in most of the media tested (LB medium, and minimal medium supplemented with glucose or glycerol with and without Casamino Acids) but caused a slight (5 to 7%) reduction in growth yields. It caused a small reduction in growth rate (less than 10%) on poor carbon sources such as succinate and acetate. The *appY*$^+$ plasmid had no effect on growth under anaerobic conditions, whether fermentative with glucose as the carbon source or via anaerobic respiration with nitrate or fumarate as the electron acceptor.

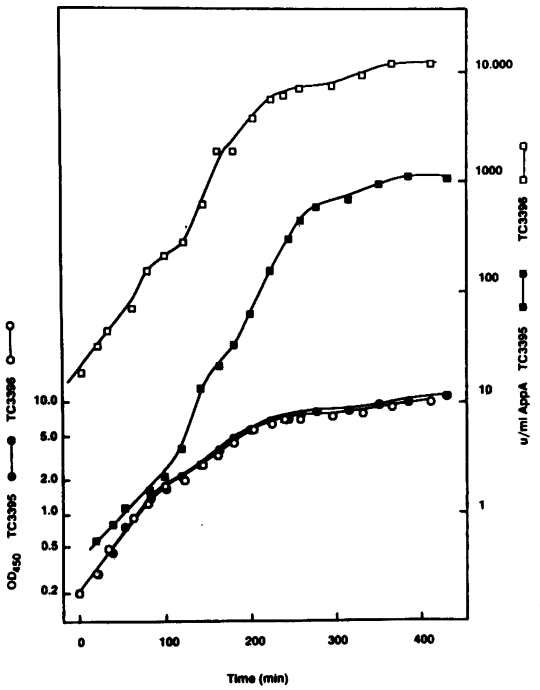

FIG. 6. Growth phase regulation of AppA activity. Strains TC3395(pUN121) and TC3396(pTAC3303) (*appY*$^+$) were pregrown exponentially in minimal AB medium supplemented with glucose and Casamino Acids for more than 15 generations and were then allowed to grow to stationary phase with good aeration. The growth was monitored by measuring optical density at 450 nm (OD_{450}), and samples were taken at intervals for determination of AppA activity.

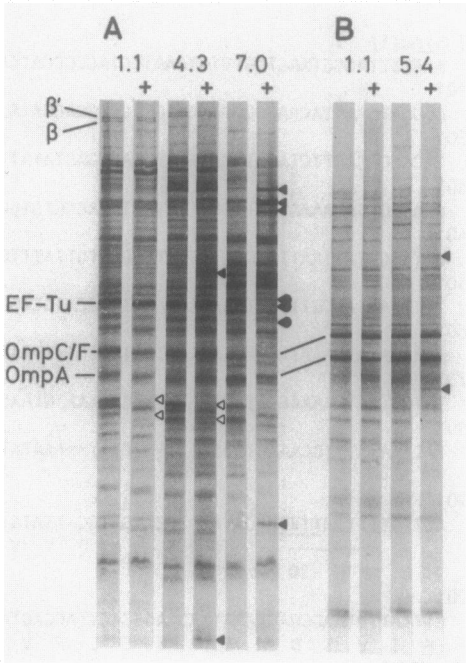

FIG. 7. Effect of AppY overproduction on synthesis of other proteins. Strains TC3395(pUN121) and TC3396(pTAC3303) (*appY*$^+$) were grown exponentially in AB medium supplemented with glucose and FN19 amino acids mixture for more than 10 generations and then allowed to grow to stationary phase. Samples were pulse-labeled with [^{35}S]methionine for 3 min at different cell densities. The polypeptides of whole-cell lysates (A) or purified membranes (B) were separated by one-dimensional sodium dodecyl sulfate-gel electrophoresis and visualized by autoradiography. The optical density at 450 nm at the time of the pulse is indicated at the top. +, Strain TC3396 carrying the *appY*$^+$ plasmid. The patterns of protein synthesis were identical from optical densities of 0.2 to 2.0. Examples of proteins affected by AppY overproduction are indicated with different arrowheads (see text), and the positions of some reference proteins are indicated to the left.

The only pronounced effects of the plasmid were a significant increase in the lag phase (of several hours) after dilution of a stationary-phase culture into fresh medium and an increased growth rate in glucose medium supplemented with a synthetic amino acid mixture.

Effect of the *appY*$^+$ plasmid on growth phase induction of AppA. The synthesis of acid phosphatase is induced in wild-type *E. coli* strains in the deceleration phase of growth in a batch culture (11).

To learn more about the effect of the *appY* product, we monitored the activity of acid phosphatase in cultures going from the exponential growth to the stationary phase. The experiment was carried out with a control strain (TC3395) carrying the vector plasmid pUN121 and with a strain (TC3396) carrying the *appY*$^+$ plasmid pTAC3303 (Fig. 6), grown in AB minimal medium supplemented with glucose and Casamino Acids. We observed a biphasic induction in both strains. The first induction occurred when the growth rate was decreased by a factor of 2, and the next occurred upon entry into the growth deceleration phase (Fig. 6). In the strain carrying the *appY*$^+$ plasmid, the rate of synthesis was 100-fold higher in the exponential growth phase and was stimulated less than in the control strain at the two induction points, such that the rate of synthesis was only 10-fold higher in the deceleration phase. In both strains, synthesis stopped upon entry into the stationary phase. Essentially the same synthesis patterns were seen when AB minimal glucose medium supplemented with synthetic amino acid mixture was used, when glycerol was substituted for glucose, or when LB medium was used.

Effects of *appY* on overall protein synthesis patterns as a function of growth phase. Strains TC3395 and TC3396, carrying the vector and the *appY*$^+$ plasmid pTAC3303, respectively, were grown in a glucose-synthetic amino acid mixture, and samples were taken at intervals over the full growth curve and pulse labeled with [^{35}S]methionine. The pattern of protein synthesis was analyzed by one-dimensional gel electrophoresis of total protein and of partially fractionated cells (Fig. 7). This showed that the synthesis of more than 30 distinguishable polypeptide bands was affected by the presence of the *appY*$^+$ plasmid. An effect similar to that on the synthesis of acid phosphatase was observed for several polypeptides, i.e., proteins induced in the deceleration phase in the control strain showed an earlier and/or enhanced induction in the strain with the *appY*$^+$ plasmid (Fig. 7, solid arrowheads). There were, however, also many protein bands that were affected differently by the *appY*$^+$ plasmid. First, a number of proteins, which were synthesized preferentially in exponential growth in the wild type, were strongly repressed by the presence of the *appY*$^+$ plasmid (Fig. 7, open arrowheads). Among these was a very prominent periplasmic protein of 25 kDa, whose synthesis was shut off in the wild type only upon entry into the stationary phase, but which was not synthesized in the *appY*$^+$ plasmid-carrying strain. The last class consisted of about 10 proteins synthesized only in the stationary phase, i.e., starvation proteins, which showed strongly reduced expression in the AppY-overproducing strain (Fig. 7, rounded solid arrowheads). Proteins from all three fractions

analyzed, i.e., membrane, periplasmic, and cytoplasmic proteins, were represented in the three classes with respect to *appY* effects. More than half of the polypeptides induced in the deceleration phase of growth were not affected visibly in the rate of synthesis or induction point in the *appY*$^+$ plasmid strain, and there was no effect on synthesis of RNA polymerase β and β′ subunits and translation elongation factors EF-G and EF-Tu.

DISCUSSION

In this study we have described the isolation and mapping of a gene designated *appY*. The *appY* gene cloned in a multicopy plasmid leads to increased expression of the *appA* gene, which codes for an acid phosphatase. The clone with the *appY* gene was isolated fortuitously when a gene library was being screened for clones carrying the *appA* gene and hence giving increased levels of acid phosphatase. We subsequently showed that *appY* affects the synthesis of a number of other proteins that show variation in the rate of synthesis as a function of growth phase.

The *appY* gene was mapped to 13 min on the current genetic map of the *E. coli* chromosome (3).

The position of the *appY* gene on the cloned 4.9-kb *Hin*dIII fragment was determined by Tn*5* mutagenesis and BAL 31 deletion analysis. The *appY* gene was found to code for a weakly expressed polypeptide migrating with an apparent molecular mass of 35 kDa by maxicell analysis of the clones. The nucleotide sequence of a 1.9-kb region showed that an open reading frame coding for 243 amino acids was contained within the region delineated for the *appY* gene. This open reading frame is preceded by a ribosome-binding site (Fig. 5, SD). We conclude that this is the *appY* gene product. The sequence of the *appY* gene region is extremely rich in A · T base pairs, and the coding region shows an unusually extreme preference for codons found primarily in genes for weakly expressed regulatory proteins (27).

Several homologies to the consensus sigma-70 promoter sequences (14) are found within the 300-bp region preceding the *appY* gene, which, according to the BAL 31 deletion analysis, contains the promoter. The promoter sequence which is located immediately before the gene shows the best fit, having TTGACT in the −35 position and TTTTAT or TATGAT in −10 (the latter would produce an mRNA lacking the SD sequence). This possible promoter contains in positions −85 to −50 a 10-bp inverted repeat, which might be a binding site for a regulatory protein.

We found by comparison of DNA sequences that the nucleotides from positions 1290 to 1874 in the *appY* sequence were identical to nucleotides 2034 to 1450 in the *ompT* sequence (20), and consequently the 44- and 42-kDa polypeptides we observed in the maxicell experiments must be the outer membrane protease encoded by the *ompT* gene (19). DNA sequence comparison also showed that the *appY* gene encodes the polypeptide called M5 (25), which has been implicated in the control of the enzymes of polysaccharide biosynthesis in *lon* strains (16). The sequence of the M5 coding region published by Kemp et al. (25) is identical to our *appY* gene sequence, except for a seven-codon insertion present in the M5 sequence at position 1002 in Fig. 5.

We have shown by use of operon fusions that the effect on acid phosphatase synthesis is at the transcriptional level (T. Atlung, unpublished results). The AppY amino acid sequence was therefore searched for sequence similarity to a number of other proteins involved in transcriptional control of gene expression by using the diagon program (42). The amino acid sequences used in the comparison were taken from the European Molecular Biology Laboratory (EMBL) data base and included those of factors acting positively on initiation of transcription such as CRP, Fnr, and OmpR of *E. coli*; the *spo0A*, *spo0F*, *spo0B*, and *spo0H* gene products from *B. subtilis*; and the sigma factors encoded by *rpoD* and *rpoH* of *E. coli*, *rpoN* of *Klebsiella pneumoniae*, and *sigE* of *B. subtilis*. The AppY sequence was also compared with various repressors (Lac, DnaA, etc.) and transcription termination and antitermination factors (Rho, NusA, NusB, BglC, and lambda N). The analysis did not indicate any evolutionary relationship between AppY and any of these proteins. No similarity to the region conserved in sigma factors (14) was found in the AppY protein.

The only significant amino acid sequence similarity found upon inspection of the diagon outprints coincided with a probable DNA-binding domain in the AppY sequence, amino acids 149 to 168 (Fig. 5). This amino acid sequence shows the α-helix–β-turn–α-helix motif of DNA-binding domains (36) by prediction of secondary structure by the method of Chou and Fasman (9). Using the weight matrix of Dodd and Egan for evaluating DNA-binding domains (13), we found a score of 1,210 for AppY; only 3% of the ca. 2,500 proteins in a data base contained amino acid sequences giving scores above 1,200.

On the basis of these findings, we propose that the *appY* gene product is a DNA-binding protein which binds to some promoters including that of *appA* and stimulates transcription in analogy with CRP or OmpR proteins.

The finding that AppY overproduction also reduced the synthesis of a number of proteins suggests that it can also act as a repressor of transcription. We propose that the activity of the AppY protein as an activator-repressor is modulated by binding of some signal molecule whose concentration varies as a function of the metabolic state of the cell.

Acid phosphatase synthesis has previously been shown to be induced under two specific conditions, i.e., anaerobiosis and starvation for P_i (11). The *appA* gene belongs to the fermentative anaerobic stimulon as defined by Jamieson and Higgins (24), since the expression is independent of the Fnr activator protein and is reduced in a strain carrying the *pgi-2* mutation (Atlung, unpublished results). *pgi* is probably allelic to *S. typhimurium oxrC*, which has been suggested to affect the production of an intermediary metabolite involved in signal transduction in the fermentative anaerobic stimulon (24). The acid phosphatase synthesis is also independent of the *phoB*, *phoR*, and *phoM* gene products (45). The AppY protein could therefore be the so far unidentified regulatory protein (transcriptional activator) of either the fermentative anaerobic stimulon or the *phoB*-independent phosphate starvation stimulon (46). An interesting alternative is that these two stimulons are interlocked such that both anaerobiosis and phosphate starvation create the same change in the concentration of a metabolite that acts as a cofactor for the AppY protein. We are presently dissecting the relatively complex transcriptional unit which contains the *appA* gene, by isolation of the three promoters that contribute to its transcription, to study their individual regulation with respect to anaerobiosis, phosphate starvation, and activation by AppY protein. We are also trying to identify some of the other genes that are regulated by the AppY protein.

ACKNOWLEDGMENTS

We are grateful to M. Russel for providing strain K549 and to S. Koefoed for expert technical assistance in the determination of the sequence. We also thank P. Boquet for communicating the *appA*

restriction enzyme map and other information prior to publication and J. Grodberg for pointing out the identity of AppY and the M5 polypeptide.

This work was supported by grants from Christian Hansen Laboratories, the Danish Technical Science Research Council, and the Danish Center of Microbiology.

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