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Overproduction of Subunit *a* of the F_0 Component of Proton-Translocating ATPase Inhibits Growth of *Escherichia coli* Cells

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A hybrid plasmid, pKY159, carrying the promoter and the proximal region of the gene cluster for proton-translocating ATPase caused growth inhibition of *Escherichia coli* cells (K. Yamaguchi and M. Yamaguchi, J. Bacteriol. **153**:550–554, 1983). The mechanism of this growth inhibition was studied, especially in terms of the responsible gene(s). Insertion of IS*1*, IS*5*, or γδ between the promoter and the gene for a possible component of the ATPase of 14,000 daltons (14K protein) released the inhibitory effect by pKY159. Deletion of the gene for subunit *a* also released the effect. However, deletion in the gene for the 14K protein released the effect only with an additional insertion within the gene. These results suggested that overproduction of subunit *a* is closely related to growth inhibition, whereas the 14K protein is not.

Proton-translocating ATPase (H^+-ATPase) has a key function in energy transduction in cells (7, 10–12, 39). The enzyme consists of two distinct portions, F_1 and F_0. F_1 is the membrane-peripheral portion and has five different subunits, α, β, γ, δ, and ε. F_0 is the membrane-intrinsic portion and has three subunits, *a*, *b*, and *c*. F_1 and F_0 act as the ATPase and proton channel, respectively, and membranes without F_1 become leaky to protons through F_0. The genes for F_1F_0 of *Escherichia coli* are clustered in the 83-minute region on the genetic map, adjacent to the origin of DNA replication (*oriC*) (1). The nucleotide sequences of all of these genes (13, 14, 20–24, 28, 33, 38) as well as the promoter region (14, 23) have been determined, and the organization of the gene cluster as an operon has been established (8, 15, 16). By sequence analysis, a new gene coding for a protein of 14,000 daltons (14K protein) has been identified within the gene cluster (14, 24). One strong promoter, upstream from the gene cluster (23, 42), and two weak ones, within the gene for the 14K protein (42), have been reported. Although the location of the strong promoter has been determined (14, 23, 42), the precise locations of the two weak promoters in the nucleotide sequence and their significance are not clear.

Several independent studies showed that a plasmid carrying a portion of the gene cluster had inhibitory effects on growth of *E. coli* and other bacteria (17, 41, 45). This phenomenon was suggested to be due to overproduction of components of the F_0 portion, possibly leading to dissipation of the proton motive force across the membranes. However, in these studies, the plasmids used carried the *oriC* region, portions of F_1 genes, and an unidentified flanking region of the gene cluster as well as F_0 genes. Therefore, the follwing questions are still unanswered. (i) Which gene(s) is essential for the phenomenon; (ii) is overproduction of all components of F_0 responsible for the effect; (iii) is the newly identified gene for the 14K protein related to the effect? Yamaguchi and Yamaguchi (44) constructed a plasmid, pKY159, the complete DNA sequence of which is known. This plasmid carries the promoter of the *unc* operon, the entire genes of subunits *a* and *c* of F_0, and ca. 80% of the gene of subunit *b*. This plasmid, which inhibits growth of wild-type cells, can be inherited in strain NK1037, possibly because its copy number is low. Introduction of this plasmid into a wild-type strain causes a decrease in the frequency of appearance of ampicillin-resistant transformants of from 10^{-2} to 10^{-3} of that on its introduction into strain NK1037. In addition, derivatives of the plasmid with an insertion in the promoter proximal region were shown to release this effect (44).

In the present study, we analyzed the following aspects of the inhibitory effect of pKY159 on cell growth: (i) the gene(s) carried on this plasmid that is responsible for the effect and (ii) the weak promoters and their function in terms of the release of the inhibitory effect. Our results suggest that at least overproduction of subunit *a* is responsible for growth inhibition and that expression of the gene for the 14K protein is not related to the effect. At least one weak promoter exists within the gene for the 14K protein, and expression of subunit *a* from this promoter releases the effect. This promoter is located within a defined portion of the gene for the 14K protein. There may also be another weak promoter within the gene for the 14K protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Derivatives of *E. coli* K-12, strain NK1037 (Hfr *dnaA46 recA1*) harboring plasmid pKY159 (44), the *recA* derivatives of strain AN719 (*uncB402 recA*) (5) and strain AN936 (*uncE429 recA*) (9), strain W3110 (wild type), and strain N17-9 (*trp recA99 uvrA rpsL*) were used in this study. pBR322 and the other plasmids constructed previously (25, 44; H. Kanazawa, T. Kiyasu, T. Noumi, M. Futai, and K. Yamaguchi, Mol. Gen. Genet., in press) or in this study are listed in Table 1 and Fig. 1 (see also Fig. 3). Cells were grown in L broth (31) or synthetic medium (Tanaka or M9) (26) supplemented with an appropriate carbon source.

Preparation and modification of plasmids. An *E. coli* strain harboring a plasmid was grown in L broth at 37°C. When the culture reached the middle of the logarithmic phase of growth, chloramphenicol (180 μg/ml) was added for amplification of plasmids, and incubation was continued for another

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TABLE 1. Genetic complementation test with various plasmids and mutations of F_0 genes

Plasmid	Complementation[d]	
	uncB402	*uncE429*
pKY159-13[a]	+	+
pKY159-14	+	+
pKY159-15	+	+
pKY159-16	+	+
pKY159-TN8	+	+
pKY159-27	+	+
pTN1751[b]	−	−
pTN1752	−	+
pTN1753	−	+
pTN1754	−	−
pTN1761	+	+
pTN1762	+	+
pTN1766[c]	+	+
pTN1767	+	+
pTN1771	−	−
pTN1772	+	+
pTN1773	−	−

[a] Insertion points at the nucleotide sequence level in the insertion type plasmids (pKY) are shown in Fig. 2.

[b] The order of plasmids with an deletion (pTN) corresponds to that in Fig. 1.

[c] This plasmid contains an insertion as well as a deletion.

[d] Ampicillin-resistant transformants were replicated on minimal agar supplemented with succinate as the sole carbon source. Succinate plus was designated as +.

18 h. Plasmid DNA was prepared after disrupting the cells by the lysozyme-EDTA procedure (30). For deletion of a portion of pKY159, DNA of pKY159 (ca. 5 μg) was digested with appropriate endonucleases at 4°C for 60 min, and the resulting mixture of DNA fragments was religated with T4 DNA ligase. The ligated DNAs were introduced into strain W3110 *recA* or strain AN719 *recA* and incubated on L broth agar containing ampicillin (50 μg/ml). Plasmid DNAs from ampicillin-resistant transformants were prepared by a rapid procedure (2), and their structure was examined by polyacrylamide gel electrophoresis and staining with ethidium bromide. For deletion of a portion of pKY159 with the exonuclease *Bal*-31 (27), the procedure described in the instruction manual from Bethesda Research Laboratories was followed. Two batches of pKY159 (ca. 10 μg each) were digested first with *Eco*RI and then with *Bal*-31 (0.4 U) for 30 and 90 s, respectively, at 25°C. The resulting two batches were combined, and after the addition of a stop solution, they were religated with T4 DNA ligase. Plasmid DNAs from ampicillin-resistant transformants of strain AN719 obtained with these religated DNAs were prepared by the rapid procedure (2), and their structures were analyzed by polyacrylamide gel electrophoresis after digestion with appropriate restriction endonucleases.

Genetic analysis. For complementation tests, competent cells of the *recA* derivatives of strains AN719 and AN936 were prepared by cold Ca^{2+} treatment (32). About 5 μg of a plasmid DNA was introduced into the cells as described previously (32), and the cells were incubated on L broth agar containing ampicillin (50 μg/ml) at 37°C. Introduction of pKY159-16 into strain W3110 gave ampicillin-resistant transformants at a frequency of 10^{-5}, and the original plasmid was recovered. This frequency of appearance of transformants was designated as no inhibition of cell growth. On the other hand, in the case of pKY159 the frequency was 10^{-8} or less, which was judged as indicating inhibition of cell growth. In the latter case, all of the transformants that appeared contained a modified pKY159 plasmid. The mutant strains used could not utilize succinate as the sole carbon source. Therefore, to check the transformation, we replicated the ampicillin-resistant transformants on synthetic agar medium (26) supplemented with glucose or succinate as the sole carbon source, incubated them for 2 days at 37°C, and then scored them as Suc^+ or Suc^-.

Preparation of DNA fragment and electrophoresis. A plasmid DNA was digested with appropriate restriction endonucleases and subjected to polyacrylamide slab-gel electrophoresis (5% acrylamide, 2 mm thick, 16 by 15 cm) at 200 V for 2 h. The gels were stained with 0.1% ethidium bromide, and DNA bands were located under UV irradiation and cut out of the gels. Slices containing DNA bands were homogenized, and the DNA was eluted as described previously (29).

Determination of DNA sequence. DNA fragments were phosphorylated at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled fragments were separated into single strands, and these were cleaved chemically by the procedure of Maxam and Gilbert (29). For the determination of insertion sites (pTN1766 and pTN1767), the DNA sequences of both strands of the *Hin*dIII$_2$-*Hae*II (in IS*1*) fragment were determined. For pTN1771, pTN1772, and pTN1773, plasmid DNA was digested with *Bam*HI plus *Pst*I, and 1,100- (pTN1771), 980- (pTN1772) and 1,000-base-pair (bp) (pTN1773) fragments, respectively, were recovered. These fragments were further digested with *Hha*I, and the DNA sequences of the resulting 450- (pTN1771), 300- (pTN1772), and 350-bp (pTN1773) fragments were determined.

Protein synthesis in a maxicell system. *E. coli* N17-9 (18) harboring various derivatives of pKY159, pBR322, and pMCR533 was constructed. The procedures for the maxicell

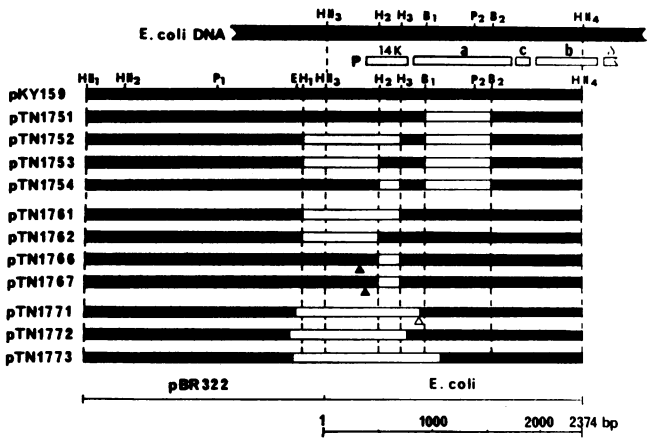

FIG. 1. Schematic illustration of plasmids with deletions. Cleavage sites with various endonucleases are abbreviated as follows: *Hae*II, HII$_1$, HII$_2$, HII$_3$, and HII$_4$; *Hin*dIII, H$_1$, H$_2$, and H$_3$; *Bam*HI, B$_1$, and B$_2$; *Eco*RI, E; *Pst*I, P$_1$ and P$_2$. At the top of the figure, *E. coli* DNA carried on plasmid pKY159 (HII$_3$-HII$_4$) and the organization of the promoter (P) and the genes for the H^+-ATPase are shown. pKY159 and all of its derivatives contain two essential *Hae*II fragments derived from the vector pBR322. The numbers on the scale at the botton of the figure correspond to those in Fig. 2. Insertions in pTN1766 and pTN1767 are shown by solid triangles. Open bars indicate portions deleted from pKY159. The portions deleted in pTN1771, pTN1772, and pTN1773 are 1,117, 948, and 1,313 bp, respectively. pTN1771 also has an insertion of 324 bp of an unknown sequence shown by an open triangle.

system were those described by Sancar et al. (37). Cells were grown in M9 medium supplemented with Casamino Acids (0.5% [wt/vol]) and tryptophan (40 μg/ml). About 1.3×10^9 cells were irradiated with UV light, and cycloserine (100 μg/ml) was added to the culture. Then, [^{35}S]methionine (10μCi, 1,390 Ci/mmol) was added, and the cultures were incubated at 37°C for 60 min. Harvested cells were lysed by freeze-thawing in the presence of lysozyme. A sample of the lysed cells (ca. 10,000 to 20,000 cpm) was subjected to electrophoresis on a polyacrylamide gel (12% acrylamide) containing sodium dodecyl sulfate and autoradiography with En^{3}Hance (New England Nuclear Corp.) for fluorography. The molecular weights of the proteins synthesized were determined with subunits of purified *E. coli* F_1 ATPase (α, 56,000; β, 52,000; γ, 31,500; δ, 20,500; ε, 12,000) as markers.

Materials. Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co. The exonuclease *Bal*-31 was purchased from Bethesda Research Laboratories. [γ-^{32}P]ATP was synthesized from $^{32}P_i$ (Amersham Corp.) and ADP by the method of Walseth and Johnson (43). The reagents used were of the highest grade commercially available.

RESULTS

Release of inhibitory effect on cell growth by an insertion into plasmid pKY159. Yamaguchi and Yamaguchi (44) reported that an insertion into plasmid pKY159 resulted in release of its inhibitory effect on cell growth. We isolated 30 derivatives of this plasmid with insertions and determined their exact insertion points at the nucleotide level. We have discussed the nature of the target structures of these insertions previously (Kanazawa et al., in press). Here we summarize the effects of these insertions into pKY159 at the points shown in Fig. 2. The insertions in pKY159-13 (IS*1*),

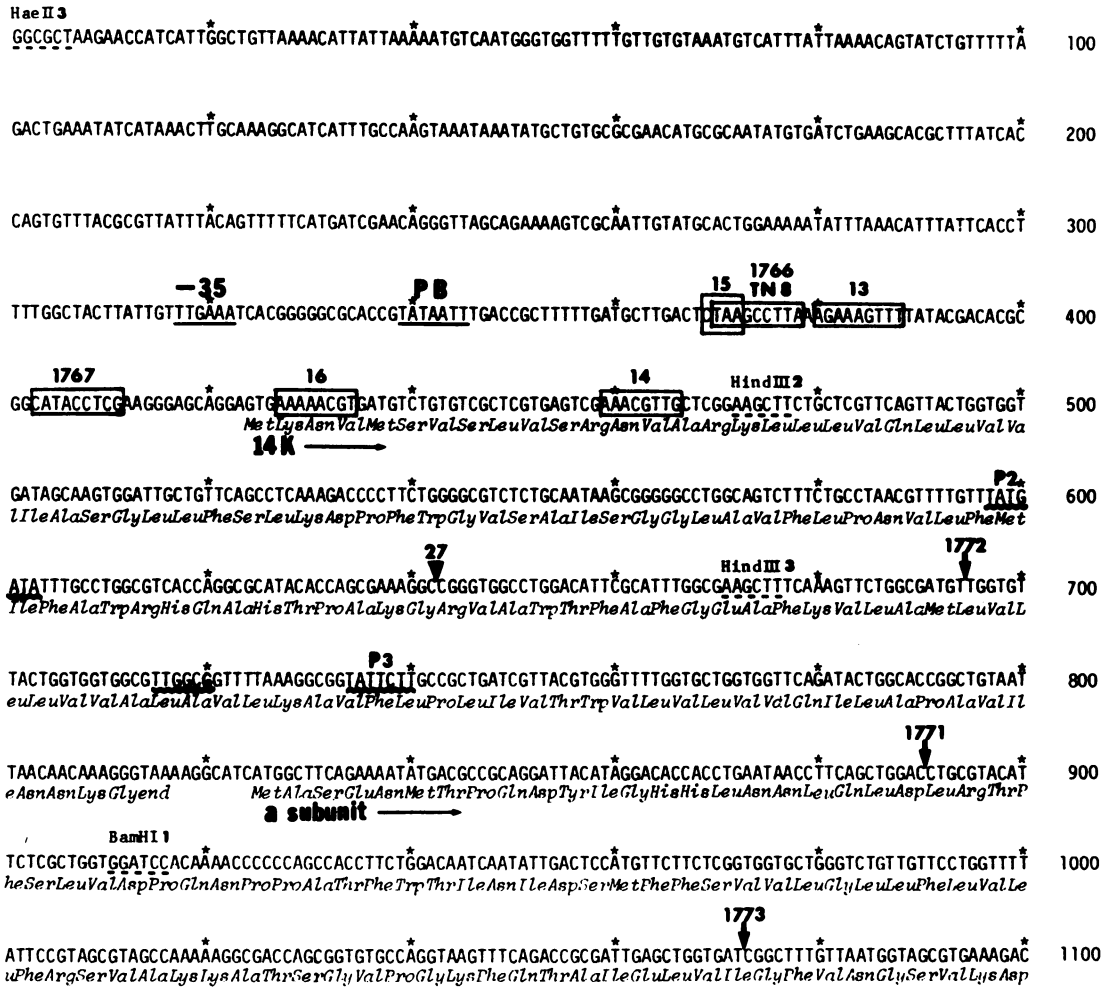

FIG. 2. DNA sequence of the promoters and promoter proximal region of the gene cluster for the H^+-ATPase and the insertion sites. The nucleotide sequence is cited from previous studies (20), and the antisense strand alone is presented. Nucleotide residues are aligned and numbered from nucleotide residue G of the *Hae*II$_3$ site. The numbers correspond to the scale shown at the bottom of Fig. 1. −35 and PB indicate the −35 region and Pribnow box for RNA polymerase, respectively. Possible promoters deduced from the nucleotide sequence are underlined with a wavy line and designated as P_2 and P_3. For P_3, a −35 region (at ca. 720 bp) is also indicated by a wavy line. See the text for details of promoters. The amino acid sequence of the 14K protein and subunit *a* is shown under the nucleotide sequence. Insertion points of IS*1* in pTN1766 and pTN1767, shown in boxes, were determined by the procedure of Maxam and Gilbert (29) in this study. Other target sites of insertions shown in boxes were determined previously (Kanazawa et al., in press). pTN1771 and pTN1772 had deletions of the sequence up to the respective points shown by arrows. Cleavage sites by restriction endonucleases are shown by dotted lines.

pKY159-TN8 (IS*1*), and pKY159-15 (IS*5*) were between the Pribnow box determined previously and the gene for the 14K protein; the insertions in pKY159-16 (IS*1*), pKY159-TN17, pKY159-14 (IS*1*), and pKY159-27 (γδ) were within the gene for the 14K protein. Introduction of these plasmids into wild-type cells did not result in growth inhibition. The results suggest two possibilities for the release of growth inhibition by the derivatives of pKY159. First, loss of expression of all of the genes carried on pKY159 may lead to release of growth inhibition. Second, expression of the 14K protein gene alone may be responsible for the growth inhibition. The latter possibility was suggested by the fact that the insertion frequency was highest at the site observed in pKY159-16, i.e., the first amino acid residue of the gene for the 14K protein (Kanazawa et al., in press).

Deletion of the gene for subunit *a* from pKY159 releasing its growth inhibition. To determine whether subunit *a* is related to growth inhibition, we prepared a derivative of pKY159 (pTN1751) with a deletion of ca. 80% of the gene for subunit *a* (the region between *BamHI*$_1$ and *BamHI*$_2$, Fig. 1). Introduction of this plasmid into a wild-type strain did not cause any growth inhibition. Plasmid DNA was recovered from the ampicillin-resistant transformant, and analytical gel electrophoresis showed that it had exactly the same structure as that of plasmid DNA introduced into wild-type cells (data not shown). These results strongly suggest that overproduction of subunit *a* caused growth inhibition.

Deletion of the gene for 14K protein from pKY159 not releasing growth inhibition. To delete the gene for the 14K protein to determine its effect on growth inhibition, we digested pKY159 with *Hin*dIII, religated the resulting mixture of DNA fragments, and introduced them into the wild-type strain. Plasmid DNAs obtained from six ampicillin-resistant transformants were analyzed and classified mainly into two types represented by pTN1761 and pTN1762 (Fig. 1), which had a deletion in the *Hin*dIII$_1$-*Hin*dIII$_3$ region (the promoter [23] and most of the gene for the 14K protein) and the *Hin*dIII$_1$-*Hin*dIII$_2$ region, respectively. We did not obtain a plasmid with a deletion of the *Hin*dIII$_2$-*Hin*dIII$_3$ fragment (most of the gene for the 14K protein) without any additional insertion; pTN1766 and pTN1767 had the deletion but also an additional insertion of IS*1*. The insertion points in these plasmids were determined from the nucleotide sequences (Fig. 2). None of the plasmids described above caused growth inhibition when introduced into the wild-type strain.

Expression of genes carried on the plasmids. The effects of insertions and deletions on gene expression by plasmid pKY159 were analyzed by genetic complementation tests. Here we used mutant strains AN719 (*uncB402 recA*) and AN936 (*uncE429 recA*), which are defective in subunits *a* and *c*, respectively. Since all of the plasmids used were derivatives of pKY159 and we have previously shown that pKY159-16 lacking the carboxyl-terminal portion of the gene for subunit *b* did not complement a mutation in the gene for subunit *b* (KF8) (34), complementation with a mutation affecting this subunit was not examined. The deletion-type plasmids (pTN1751, pTN1752, pYN1753, and pTN1754) defective in the subunit *a* gene did not complement the mutation as expected. On the other hand, unexpectedly, the insertion-type plasmids (pKY159-13, pKY159-14, pKY159-15, pKY159-16, pKY159-27, pKY159-TN8, pTN1766, and pTN1767) complemented the *uncB402* mutation, suggesting that the gene for subunit *a* is expressed in these cases. It is known that an insertion of IS*1* results in a severe polar effect on the expression of genes located distal to the insertion with respect to the promoter proximal region (19). Therefore, it is likely that these plasmids are defective in transcription of the *unc* operon from the promoter (P1, Fig. 2). The directions of IS*1* insertion in pKY159-TN17 and pTN1766 are opposite to those of IS*1* insertion in pKY159-16 and pKY159-TN8, respectively, which have IS*1* at the same positions (data not shown). These plasmids (pKY159-TN17 and pTN1766) also complemented the *uncB402* mutation. These results suggested that another promoter(s) exists downstream from the insertion sites in addition to the promoter discussed above (P1, Fig. 2). This notion was confirmed by the results described below.

The *uncE429* mutation, defective in subunit *c*, was complemented by plasmids pTN1752, pTN1753, pTN1766, pTN1767, and all of the plasmids with an insertion (Table 1, and Fig. 2). However, unexpectedly, the same mutant was not complemented by pTN1751 or pTN1754 (Table 1, and Fig. 2). The two groups of plasmids obviously differ in that the former contains the *Hae*II$_3$-*Hin*dIII$_2$ region, and the latter group does not (including an insertion). It is unknown why pTN1751 and pTN1754 did not complement the mutation. Possibly the existence of the *Hae*II$_3$-*Hin*dIII$_2$ region with a deletion of the *Bam*HI$_1$-*Bam*HI$_2$ region affects the expression of the gene for subunit *c*. As discussed below, there is another promoter in the *Hin*dIII$_3$-*Bam*HI$_1$ region in addition to the one determined previously (P1, Fig. 2) (23). The presence of these two promoters without a functional gene for subunit *a* may lead to a decrease in expression of subunit *c*.

A promoter within the gene for 14K protein. The existence of a promoter within or downstream from the gene for the 14K protein has been suggested. It was located as follows. The unique *Eco*RI site in plasmid pKY159 (Fig. 2) was cut by the enzyme, and the flanking region of the site was deleted with the exonuclease *Bal*-31. The resulting mixture of DNAs was introduced into a mutant (*uncB402*), and then plasmids (pTN1771, pTN1772, and pTN1773) (Fig. 2) were recovered from three transformants. The deleted portions in these plasmids were determined by sequencing the plasmid DNAs (Fig. 2). pTN1771 and pTN1773 did not complement the *uncB402* mutation but pTN1772 did. Since pTN1772 has a 218-bp region of the *Hin*dIII$_3$-*Bam*HI$_1$ fragment and pTN1771 has a 23-bp flanking region of the *Bam*HI$_1$ site, the DNA segment of 195 bp within the *Hin*dIII$_3$-*Bam*HI$_1$ region (Fig. 1) is essential for expression of the gene for subunit *a*, suggesting that this segment carries a promoter.

Synthesis of subunit *a* directed by the plasmids in a maxicell system. The synthesis of subunit *a* was detected in the maxicell system with plasmids pKY159-13, pKY159-16, pKY159-TN8, and pTN1772 but not with pTN1751 and pTN1771, which had a deletion of the gene for subunit *a* (Fig. 3). When the 14K protein is synthesized, a band should be visible near the region corresponding to the ε subunit. Since all of the plasmids analyzed (Fig. 3) have an insertion or deletion within or upstream from the gene for the 14K protein, no synthesis of the protein was expected. In fact, such a band was not observed, suggesting that the 14K protein was not synthesized in any case. In this connection, it should be noted that expression of the gene for 14K protein from the promoter (P1, Fig. 2) has been demonstrated in an in vitro transcription-translation system and a minicell system (4). However, the synthesis of 14K protein directed by pKY159 could not be demonstrated in the maxicell system, because this plasmid has a lethal effect for cells used in this system. Although subunit *c* should be synthesized because all of the plasmids used except pTN1751 complemented the

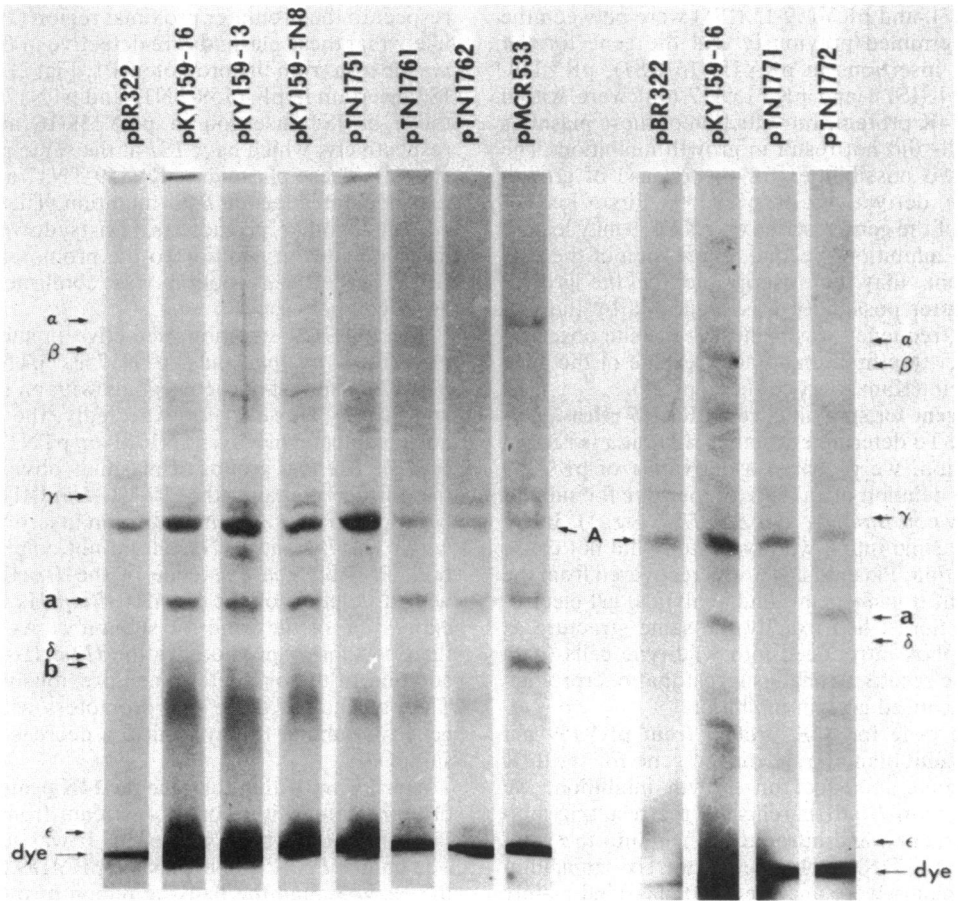

FIG. 3. Synthesis of polypeptides directed by the plasmids in a maxicell system. Strains (derivatives of N17-9) harboring plasmids used in this experiment were constructed as described in the text. About 1.3×10^9 cells were irradiated with UV light, and [^{35}S]methionine was added. A sample of lysed cells was subjected to polyacrylamide gel electrophoresis and subsequent autoradiography. The band corresponding to a molecular weight of 30,000 (A) is that of β-lactamase coded by the vector plasmid. The subunits of *E. coli* F_1 ATPase shown were used as standard molecular weight markers.

uncE mutation, it was hard to detect the band of subunit *c* because it migrated to the dye front on gel electrophoresis.

DISCUSSION

The results indicated that the insertions into a plasmid pKY159 released the growth inhibition of *E. coli* cells by this plasmid, raising two possibilities for growth inhibition. Either the expression of all of the genes (14K protein, subunits *a* and *c*, and 80% of subunit *b*) or only that of the gene for the 14K protein is responsible for the growth inhibition. The latter possibility seems unlikely for the following reasons. When we tried to construct plasmids by deleting several DNA fragments with the *Hin*dIII site at the two ends to destroy the gene for the 14K protein, only plasmids (pTN1766 and pTN1767) with an additional insertion in addition to the expected deletion in the middle of the gene for the 14K protein were obtained; i.e., no deletion plasmids without insertions were obtained. Therefore, insertions were requisite for release of the growth inhibition. Since a 199-bp portion of the gene for the 14K protein (390 bp) was deleted in this experiment and an additional insertion was required in pKY159 for the release of the effect, we concluded that the 14K protein is not related to growth inhibition. In this connection, the following findings should be noted. Although the 14K protein was not found in the purified F_1F_0, it has recently been shown that this protein can be synthesized in the minicell system (4). Therefore, this protein should exist in *E. coli* cells. von Meyenburg et al. (42) reported that the insertion of Tn*10* into the gene for the 14K protein did not bring about total loss of the function of F_1F_0, suggesting that this protein is not indispensable for function of the F_1F_0 complex.

The deletion of a 617-bp central portion of the gene for subunit *a* (813 bp) from pKY159 abolished growth inhibition by this plasmid, clearly indicating that subunit *a* is responsible for the growth inhibition. However, the plasmids with an insertion, such as pKY159-16, complemented the *uncB402* and *uncE429* mutations, suggesting that the subunit proteins were synthesized. The synthesis of subunit *a* was confirmed directly in a maxicell system. These results indicate that growth of cells was not inhibited under conditions in which the synthesis of subunit *a* was directed by the derivative plasmids of pKY159 (pKY159-13, pKY159-14, pKY159-15, pKY159-TN8, pKY159-27, pTN1766, and pTN1767). Therefore, it seems that overproduction of subunit *a* at above a certain level inhibits growth of cells, whereas slight overproduction of the subunit does not. It would be interesting to know the minimal level of expression of subunit *a* for growth

inhibition. Yamaguchi and Yamaguchi (44) reported that pKY159 was inherited in strain NK1037 because in this strain the copy number of the plasmid was low. This observation is consistent with our observation on the effect of subunit *a*. Although the inhibitory effects of plasmids carrying portions of the gene cluster for F_1F_0 on cell growth were reported by several workers (17, 41, 45), the plasmids used in these studies contained not only F_0 genes but also the *oriC* region or F_1 genes or both. Thus the results in the present study are the first evidence of the involvement of subunit *a* in this phenomenon.

von Meyenburg et al. (42) suggested that overproduction of F_0 components may lead to dissipation of the proton motive force across the membrane, resulting in growth inhibition of the cells. However, no clear evidence for this has thus far been presented in terms of the mechanism involved, although von Meyenburg et al. reported that dicyclohexylcarbodiimide, which prevents leak of protons through F_0, released the growth inhibition caused by a plasmid carrying a portion of the gene cluster of F_1F_0. On the other hand, it was found that a functional assembly of the F_0 portion requires at least coordinate synthesis of the β subunit of F_1 (6). However, it should be noted that pKY159 does not carry any genes for F_1 components and even carries a defective gene for the *b* subunit. Since we have shown that pKY159-16 did not complement strain KF8 defective in subunit *b* (34), incomplete subunit *b* coded by pKY159 and its derivatives may be functionally defective. Therefore, it is unlikely that overproduction of functional F_0 took place in cells harboring pKY159, resulting in growth inhibition. Overproduction of subunit *a* itself may be harmful for growth of the cells. For instance, unbalanced production of subunit *a* may affect the assembly of F_0. Plasmid pTN1751 with a deletion of subunit *a* did not complement a mutation in subunit *c* (*uncE429*). Therefore, it is uncertain whether subunit *c* is necessary for growth inhibition. For further elucidation of the mechanism of growth inhibition, the effect of overproduction of subunit *c* and an incomplete subunit *b* directed by pKY159 should also be analyzed.

As described above, the level of expression of the gene for subunit *a* seems to differ in the case of pKY159 and its derivatives with an insertion, such as pKY159-16, suggesting the existence of a promoter with low activity in addition to the one reported previously (23). In fact, a region with activity as a promoter was located within the *Hin*dIII$_3$-*Bam*HI$_1$ region. Judging from the nucleotide sequence, there may be a Pribnow box (P3 in Fig. 2) in this region (Fig. 2). von Meyenburg et al. (42) reported two weak promoters (second [P2] and third [P3] promoter) within the gene for the 14K protein and one strong promoter (first promoter [P1]) upstream from the gene cluster, which seems to be the same as that which we described previously (24). The promoter (P3) we described in this study may correspond to the third one described by von Meyenburg et al. (42), although they did not indicate its precise location in the nucleotide sequence. Consistent with our findings, Porter et al. also recently found weak promoters within the gene for the 14K protein (35).

Since there is more than one potential promoter (P2 and P3) in the gene for the 14K protein, the expression of the subunit *a* gene may depend on a promoter (P2, Fig. 2) other than that in the *Hin*dIII$_3$-*Bam*HI$_1$ region (P3, Fig. 2) in the case of insertion-type plasmids pKY159-13, pKY159-14, pKY159-16, pKY159-15, and pKY159-TN8. However, in the case of plasmids which carry regions downstream from *Hin*dIII$_3$ (pTN1761 and pMCR533), it is reasonable to assume that the gene for subunit *a* is expressed from the promoter (P3) found in this study. It is known that a suitable promoter in the vector plasmid pBR322 (40), which produces a read-through, does not exist (3; H. Kanazawa unpublished data) when a DNA is introduced into a unique *Hin*dIII site in the vector. The results with the maxicell system indicated that the production levels of subunit *a* by insertion-type plasmids were significantly lower than that of β-lactamase, whereas production levels by plasmids carrying regions downstream from *Hin*dIII$_3$ were about the same or slightly lower than that of β-lactamase (Fig. 3). These observations suggest that in the two different types of plasmids the gene for subunit *a* was expressed from two promoters, possibly the second (P2) and third (P3) promoters with different efficiencies, and also that the third promoter (P3) alone has stronger activity than that of the second (P2) and third (P3) ones together. The estimated activities of the two promoters are not consistent with the report of von Meyenburg et al. (42) that the promoter activities of the first, second, and third promoters are in this order with an approximate contribution ratio of 7:2:1. The regulation of switching of these promoters in vivo is not clear. Possibly weak promoters may provide some kind of attenuation of the expression of the gene cluster for F_1F_0.

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