CHAPTER III

RESULTS

Preparation and Characterization of Plasmid DNAs

The plasmids pCSBC8 and pHV33 were extracted from *E. coli* and *B. subtilis*, respectively, and analyzed by gel electrophoresis as shown in Fig.3.1. Linear form of pCSBC8 and pHV33 were obtained after digestion with *Pst* and *Bam*HI, respectively. The size of pCSBC8 is about 9 kb (Fig.3.1 A lane c), and the size of pHV33 is about 6.8 kb (Fig.3.1 B lane c).

From the result shown in Fig.3.2, plasmid pCSBC8 was doubly digested with *Pst*I and *Kpn*I. Two DNA fragments of about 5 kb and 4 kb were obtained (Fig.3.2 A lane d). Linear form of pCSBC8 was obtained after digestion with *Hin*dIII (Fig.3.2 B lane e). However, the plasmid could not be cleaved with *Bam*HI or *Eco*RI (Fig.3.2 B lane c and d, respectively). Since the plasmid size and the DNA fragment size obtained from the restriction endonuclease digestion corresponded to its restriction map (Appendix IV), the result clearly showed that the purified plasmid is pCSBC8.

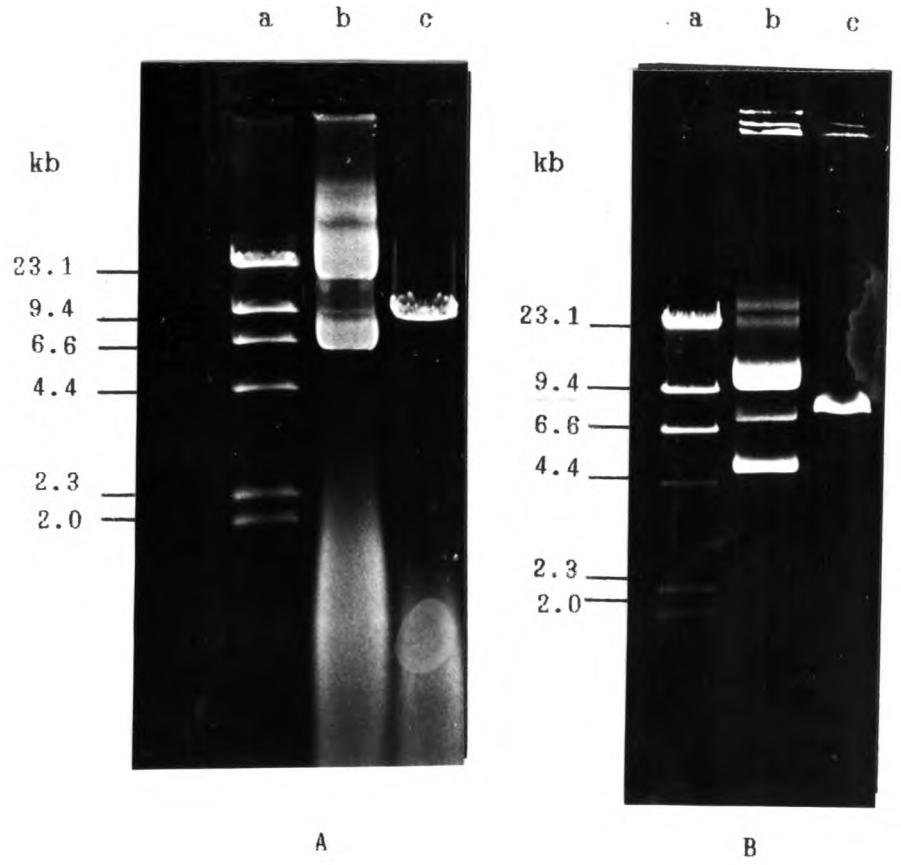


Figure 3.1 Electrophoretic analysis of plasmids pCSBC8 and pHV33

A

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Pst*I

B

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Bam*HI

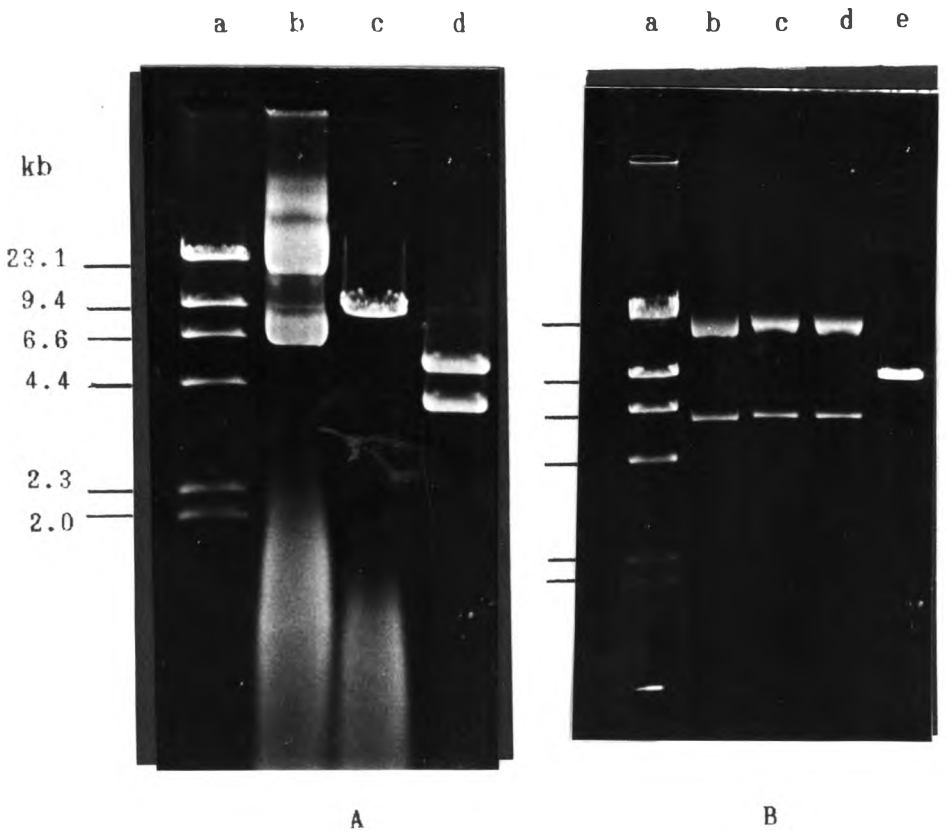


Figure 3.2 Electrophoretic analysis of plasmid pCSBC8

A

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Pst*I

lane d : pCSBC8 digested with *Pst*I and *Kpn*I

B

lane a : λ/*Hin*dIII (DNA size marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Bam*HI

lane d : pCSBC8 digested with *Eco*RI

lane e : pCSBC8 digested with *Hin*dIII

Plasmid pHV33 was also doubly digested with *Bam*HI and *Eco*RI in Fig.3.3. Two DNA fragments of about 4 kb and 2.8 kb were obtained (Fig.3.3 lane d). The results correspond to the restriction map of pHV33 (Appendix II), which indicate that the expected plasmid is pHV33.

Subcloning of the CGTase Gene from pCSBC8 to the Shuttle Vector pHV33 at *Hin*dIII and *Sal*I Sites

The experiment was to subclone the CGTase gene from pCSBC8 to the shuttle vector pHV33 at *Hin*dIII and *Sal*I sites. Subcloning would inactivated the tetracycline resistance gene in pHV33, making it possible to select the recombinant clones by replica plating.

The pCSBC8 was cleaved with restriction endonucleases *Hin*dIII and *Sal*I to excise the CGTase gene (4.7 kb) from vector DNA (pSE411, 4.4 kb) (Fig.3.4). The DNA fragments were separated by gel electrophoresis as shown in Fig.3.5.

However, the size of CGTase gene containing DNA fragment (4.7 kb) and the vector pSE411 (4.4 kb) were very close (Fig.3.5 lane d). It was difficult to separate the two DNA fragments apart. Since there was a single site of *Cla*I in pCSBC8 that was on the vector pSE411 (Appendix V), pCSBC8 was thus digested with three endonucleases *Cla*I, *Hin*dIII and *Sal*I. The fragments were separated by gel electrophoresis as shown in Fig.3.6 lane d. The 4.4 kb pSE411 fragment was cleaved into 3.4 kb and 1 kb with *Cla*I.

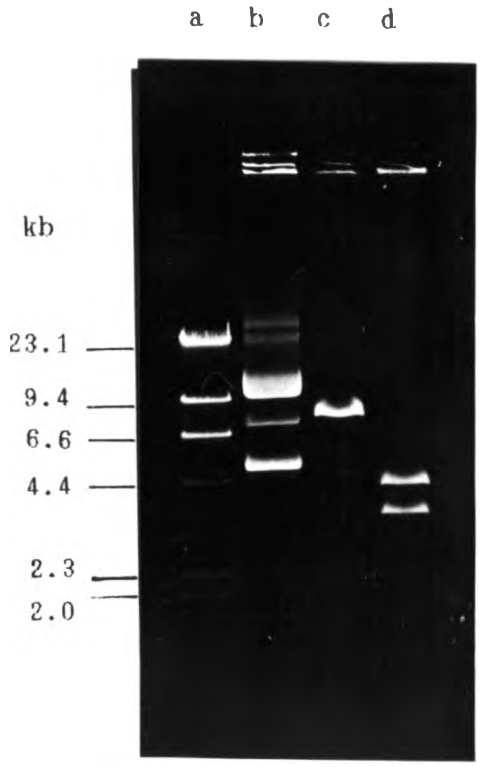


Figure 3.3 Electrophoretic analysis of plasmid vector pHV33

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Bam*HI

lane d : pHV33 digested with *Bam*HI and *Eco*RI

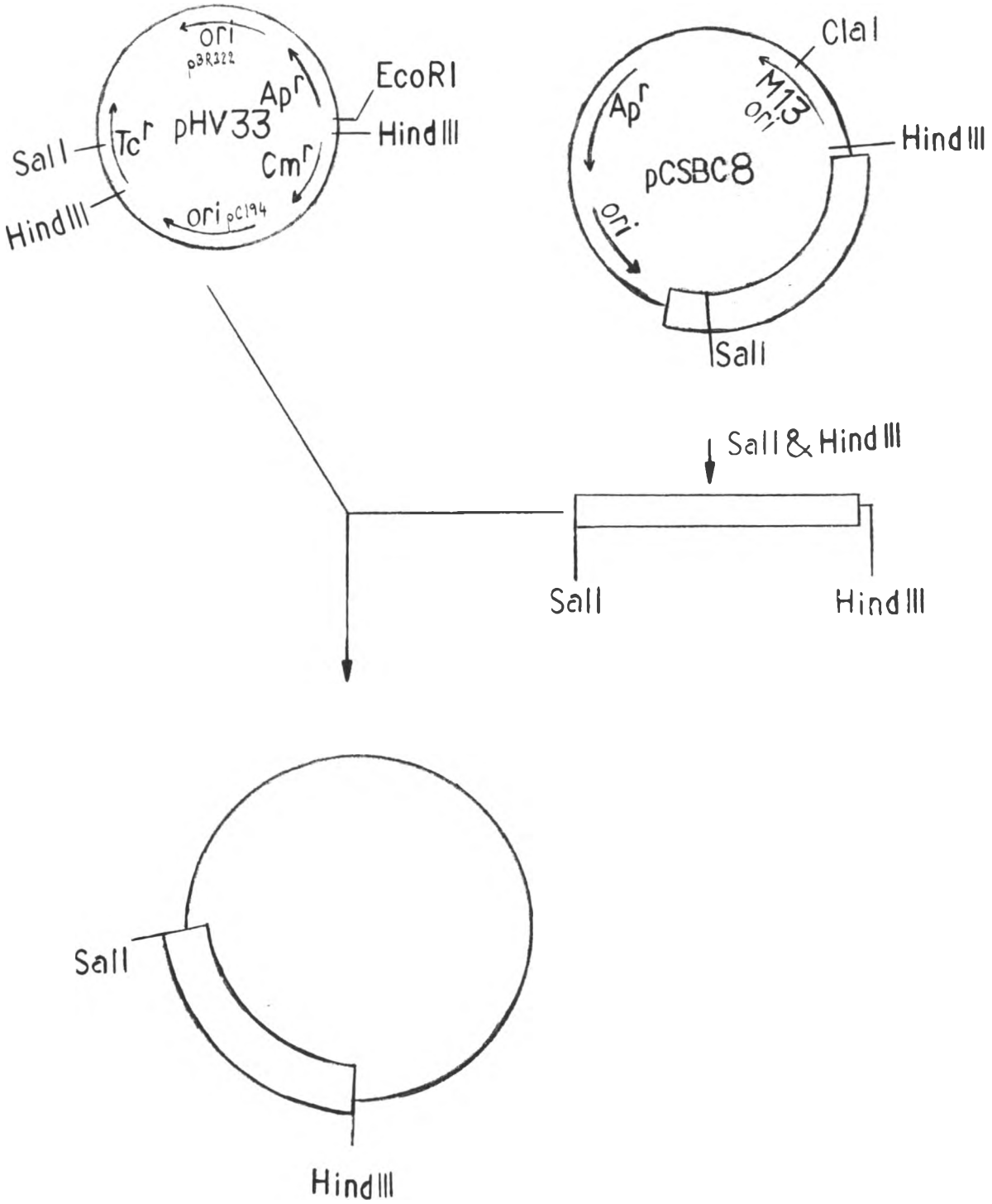


Figure 3.4 Experimental design of the subcloning of CGTase gene to shuttle vector pHV33 at *Hin*dIII and *Sal*I sites

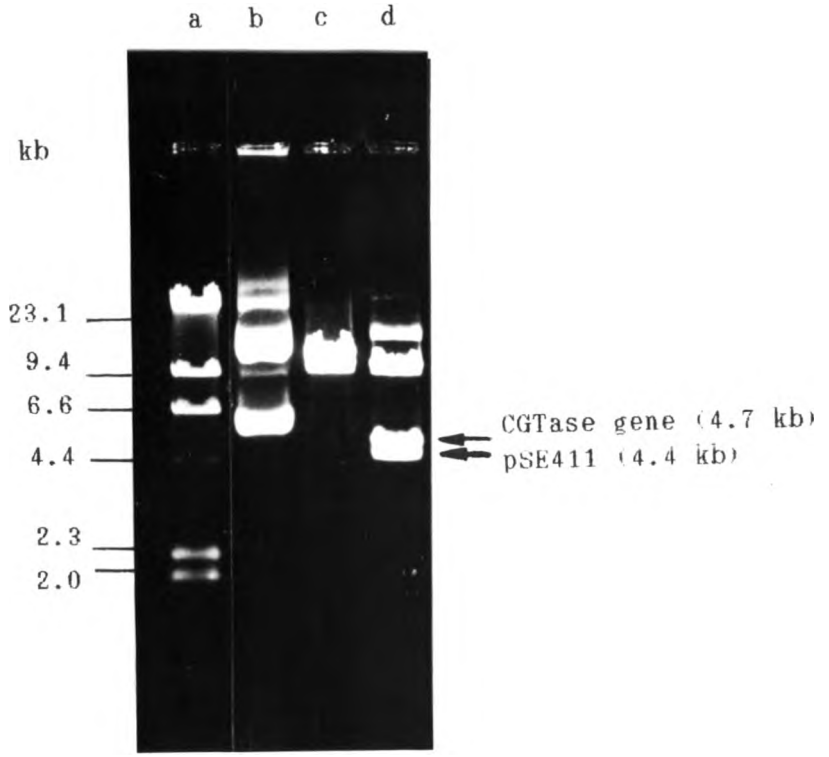


Figure 3.5 Electrophoretic analysis of pCSBC8 to demonstrate excision of CGTase gene with restriction endonucleases *Hin*dIII and *Sal*I

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Hin*dIII

lane d : pCSBC8 digested with *Hin*dIII and *Sal*I

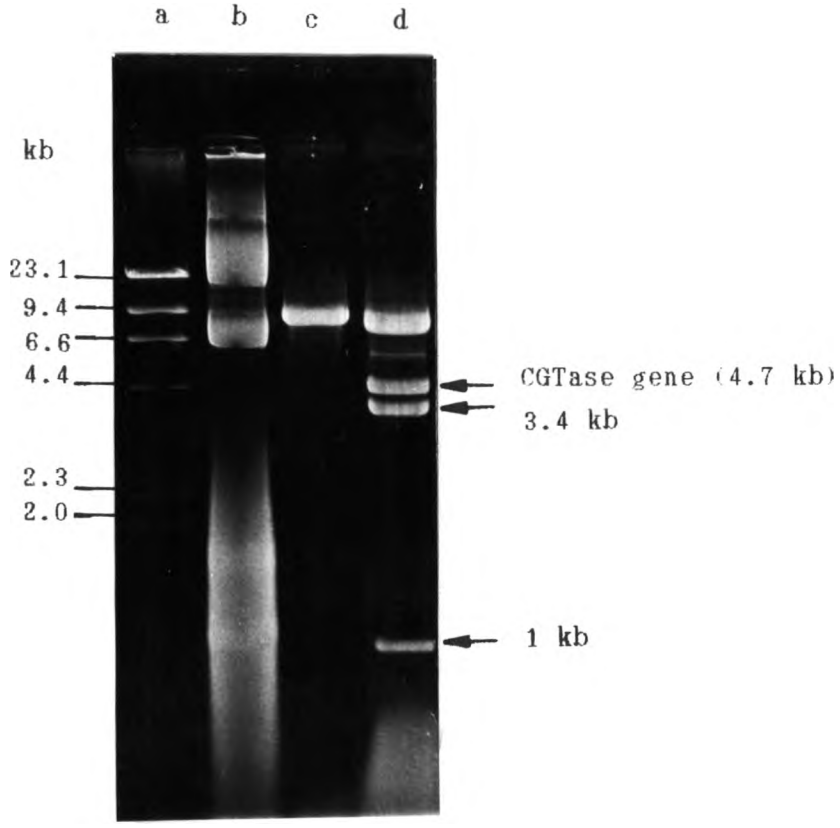


Figure 3.6 Electrophoretic analysis of pCSBC8 to demonstrate the separation of CGTase gene with restriction endonucleases, *Cla*I, *Hin*dIII and *Sal*I

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Pst*I

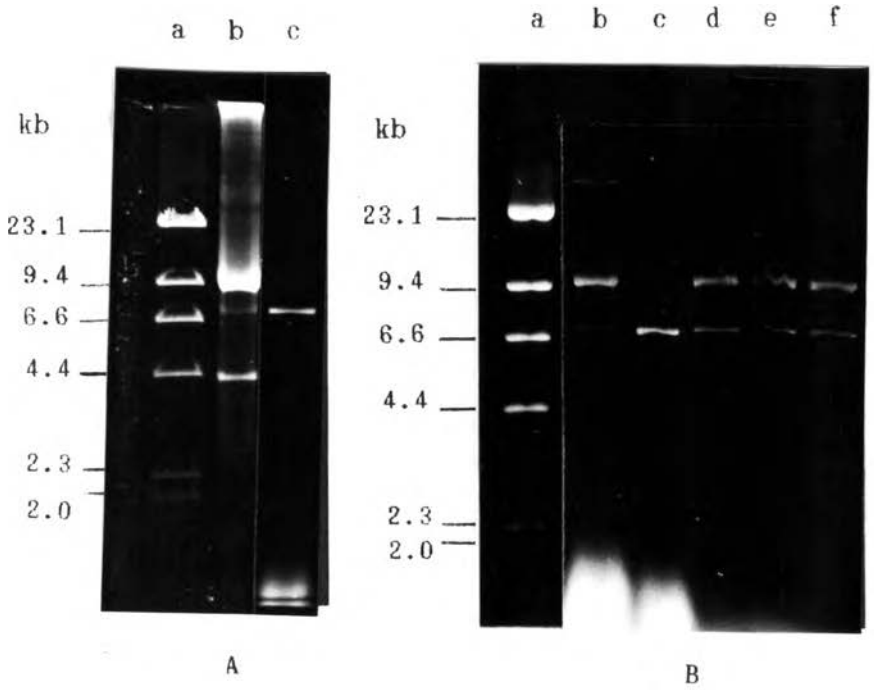
lane d : pCSBC8 digested with *Cla*I, *Hin*dIII and *Sal*I

Then CGTase gene containing fragment (4.7 kb) from pCSBC8 was recovered from agarose gel by freeze-squeeze method for further ligation with vector pHV33.

The vector DNA pHV33 was prepared by cleaving at *Sal*I and *Hin*dIII sites. There were two sites of *Hin*dIII in the vector pHV33, one site located on tetracycline resistance gene whereas the other was near chloramphenicol resistance gene (Fig.3.4). In order to prepare the linear form of vector DNA (6.2 kb) with *Sal*I and *Hin*dIII sites, located on tetracycline resistance gene, this plasmid was first completely digested with *Sal*I, and then partially digested with *Hin*dIII by varying the incubation time.

Fig.3.7 A was the result of completely digested pHV33 (lane b) with *Sal*I (lane c), the single band of open form of pHV33 (6.8 kb) was obtained. After the pHV33 had been completely digested with *Sal*I, the reaction mixture was extracted with phenol chloroform mixture, and DNA was recovered by ethanol precipitation. Then it was partially digested with 5 U *Hin*dIII at various time from 15 min to 8 h (Fig.3.7 B lane d-f, and Fig.3.7 C lane b-d). The result showed that pHV33 which had been digested with *Sal*I could not be cleaved with *Hin*dIII. Therefore the linear form of vector pHV33 (6.2 kb) could not be prepared by this order of restriction cleavage (Fig.3.7).

The vector DNA pHV33 was prepared again in order to obtain the linear form of vector DNA (6.2 kb) with *Sal*I and *Hin*dIII sites (located on tetracycline resistance gene), but this time reversed


a b c
kb
23.1
9.4
6.6
4.4
2.3
2.0
A
a b c d e f
kb
23.1
9.4
6.6
4.4
2.3
2.0
B

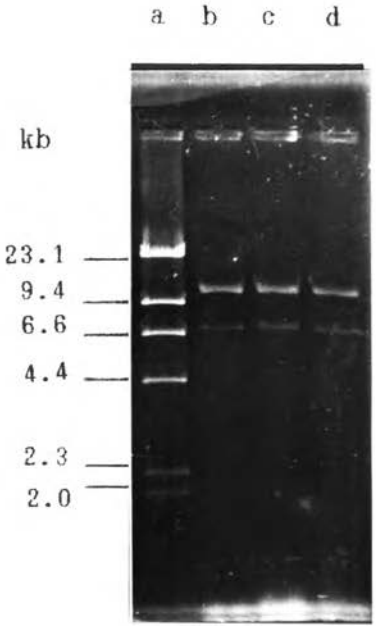

a b c d
kb
23.1
9.4
6.6
4.4
2.3
2.0
C

Figure 3.7

Figure 3.7 Electrophoretic analysis of plasmid vector pHV33, completely digested with restriction endonucleases *Sal*I and then partially digested with *Hin*dIII

A

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Sal*I

B

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Sal*I

lane d : pHV33 digested with *Sal*I and 5U *Hin*dIII for 15 min

lane e : pHV33 digested with *Sal*I and 5U *Hin*dIII for 30 min

lane f : pHV33 digested with *Sal*I and 5U *Hin*dIII for 45 min

C

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33 digested with *Sal*I and 5U *Hin*dIII for 4 h

lane c : pHV33 digested with *Sal*I and 5U *Hin*dIII for 6 h

lane d : pHV33 digested with *Sal*I and 5U *Hin*dIII for 8 h

the order of restriction cleavage. Plasmid pHV33 was first partially digested with 5 U *Hin*dIII by varying incubation time and then complete digested with *Sal*I.

After pHV33 had been partially digested with 5 U *Hin*dIII at various times from 30 min to 6 h, the reactions were stopped by extraction with phenol chloroform mixture, and recovered by ethanol precipitation. Then they were completely digested with *Sal*I (Fig.3.8 A lane b-d and Fig.3.8 B lane b-c). The result in Fig.3.8 A showed that the pHV33 which had been partially digested with *Hin*dIII for 30 min, 1 h and 2 h (Fig.3.8 A lane b, c and d, respectively), could not be cleaved with *Sal*I. However, when digested at time with *Hin*dIII, i.e. 4 h and 6 h (Fig.3.8 B lane b and c), the *Hin*dIII-digested pHV33 could be further digested with *Sal*I. The result in Fig.3.8 B also showed that the pHV33 was completely digested with *Hin*dIII within 4 h of incubation yielding two DNA fragments of 4.3 kb and 2.5 kb. Linear form of pHV33 (6.8 kb) was also seen in this digestion. The *Hin*dIII-digested pHV33 fragment of 4.3 kb was cleaved into 3.7 kb fragment with *Sal*I. From the above results, the linear form of vector pHV33 6.2 kb could not be prepared by this alternative experiment. Therefore, subcloning of CGTase gene from pCSBC8 to the shuttle vector pHV33 at *Hin*dIII and *Sal*I sites (on tetracycline resistance gene) could not be done successfully.

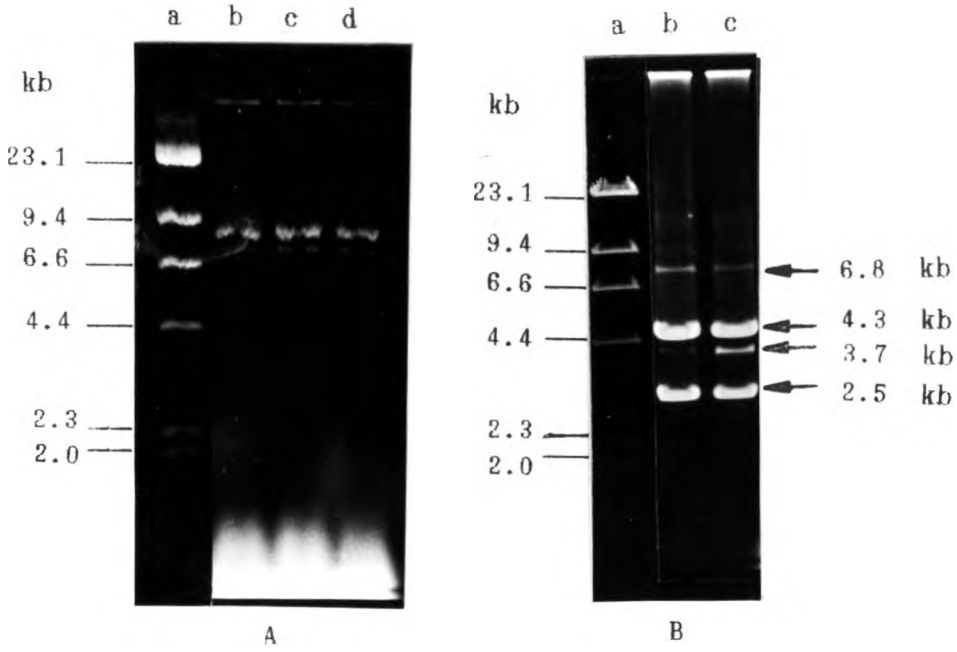


Figure 3.8 Electrophoretic analysis of plasmid vector pHV33, partially digested with restriction endonuclease *Hin*dIII and then completely digested with *Sal*I

A

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33 digested with 5U *Hin*dIII for 30 min and *Sal*I

lane c : pHV33 digested with 5U *Hin*dIII for 1 h and *Sal*I

lane d : pHV33 digested with 5U *Hin*dIII for 2 h and *Sal*I

B

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33 digested with 5U *Hin*dIII for 4 h and *Sal*I

lane c : pHV33 digested with 5U *Hin*dIII for 6 h and *Sal*I

Subcloning of pC194 Segment from pHV33 into *Hin*dIII Site of pCSBC8 and pCSBC5

This experiment was to subclone a pC194 segment from pHV33 into a *Hin*dIII site of pCSBC8 and pCSBC5. Chloramphenicol resistance gene will be added for selection in this subcloning. The pCSBC8 and pCSBC5 (Fig.3.9) were cleaved with restriction endonuclease *Hin*dIII and analyzed by gel electrophoresis as shown in Fig.3.10. The result clearly showed that both pCSBC8 and pCSBC5 were completely digested with *Hin*dIII since linear DNA fragments of 9.6 kb and 7.9 kb were obtained (lane c of Fig.3.10 A and B, respectively).

The pC194 segment was prepared by cleaving pHV33 at *Hin*dIII sites. *Hin*dIII cleavage yielded two DNA fragments of pBR322 and pC194 segments of 4.3 kb and 2.5 kb, respectively (Fig.3.11 lane c). DNA fragment of "pC194 segment" (2.5 kb) was isolated from agarose gel by freeze-squeeze method. The *Hin*dIII-digested pCSBC8 and pCSBC5 were also recovered from agarose gel by freeze-squeeze method and ligated with the "pC194 segment" from pHV33. The mixture was transformed to competent cells of *E. coli* DH5α and JM101, and the recombinant plasmids were selected on ampicillin plates. However, it was found that no transformant, carrying the hybrid plasmid, was observed.

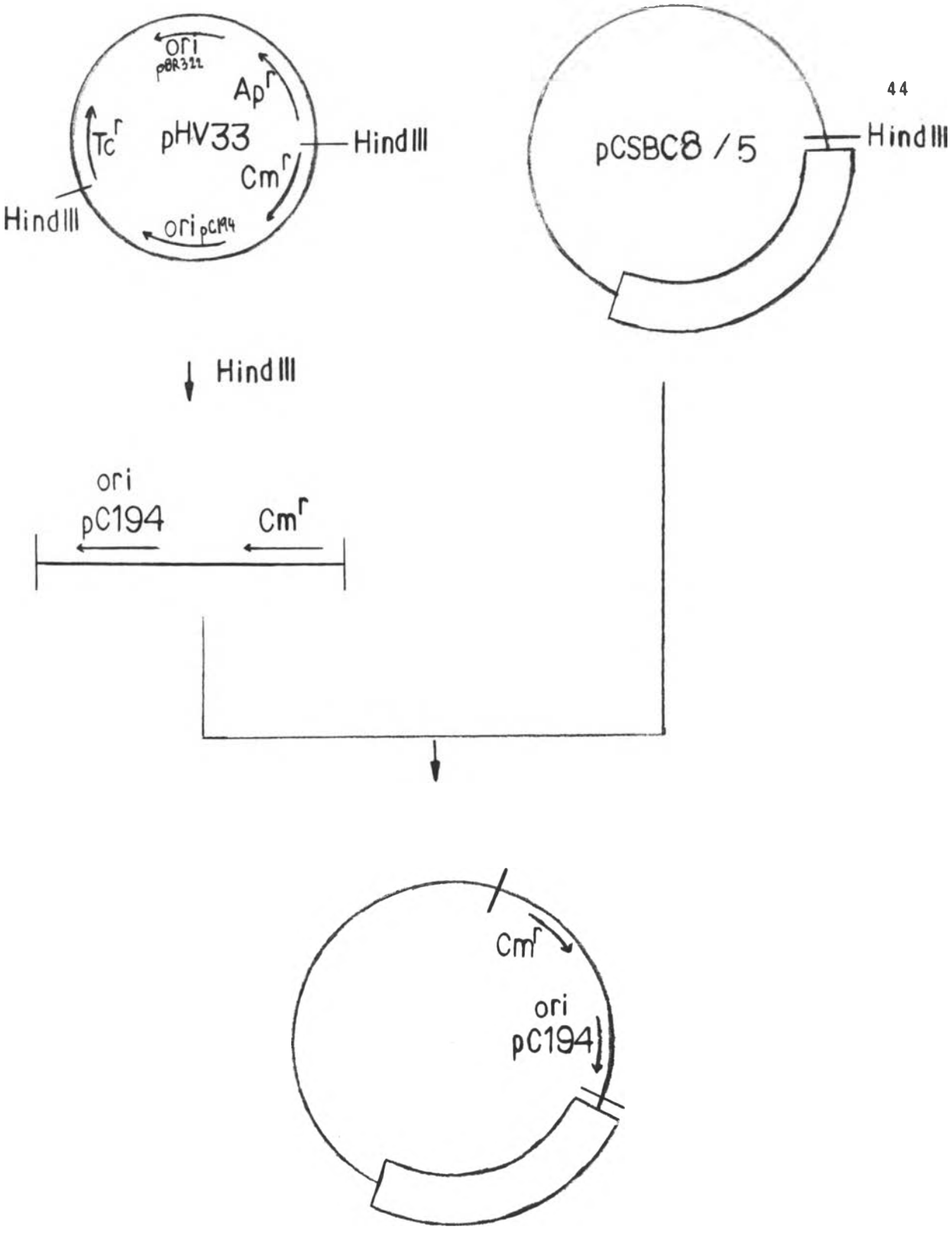


Figure 3.9 Subcloning of pC194 segment from pHV33 into *Hin*dIII site of pCSBC8 and pCSBC5

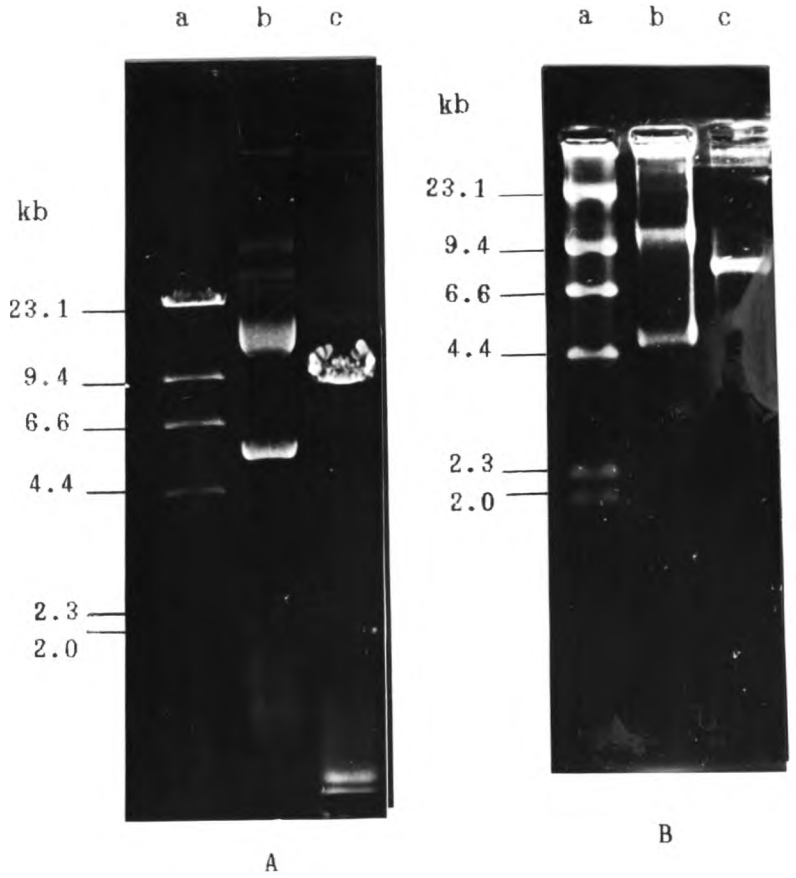


Figure 3.10 Electrophoretic analysis of *Hin*dIII-digested pCSBC8 and pCSBC5

A

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC8

lane c : pCSBC8 digested with *Hin*dIII

B

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC5

lane c : pCSBC5 digested with *Hin*dIII

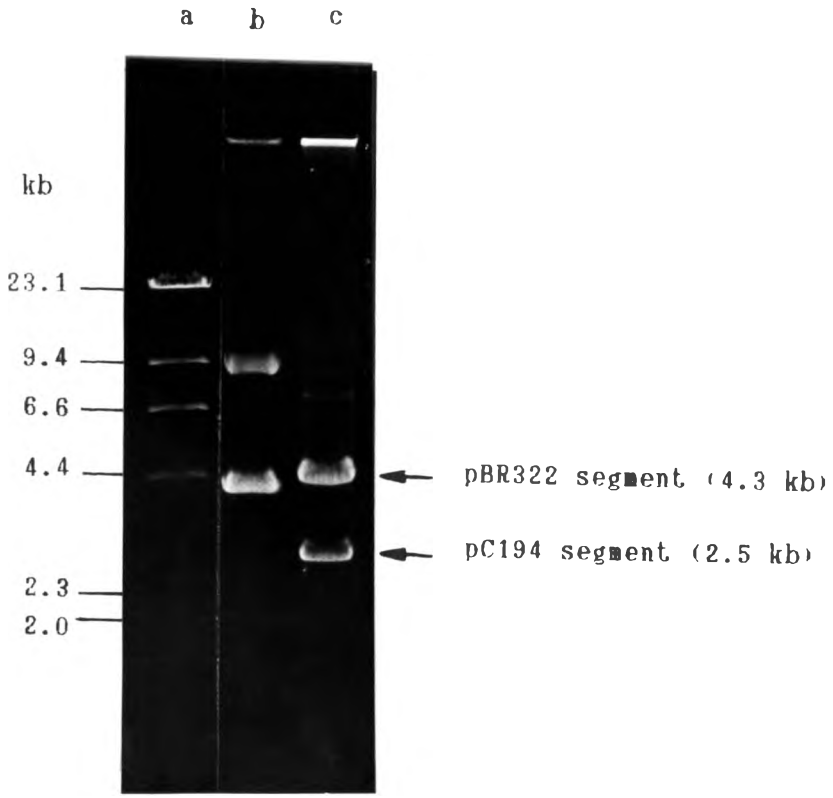


Figure 3.11 Electrophoretic analysis of *Hin*dIII-cleaved pHV33

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Hin*dIII

To understand more about the failure of the cloning,the pSE411 and pUC18 were used as controls for this experiment because they were vector DNAs in recombinant plasmids pCSBC8 and pCSBC5, respectively (the restriction map of pCSBS5 was shown in Appendix VI). The pSE411 and pUC18 were digested with *Hin*dIII and ligated with the "pC194 segment" from *Hin*dIII-cleaved pHV33. The mixture was transformed to competent cells of *E. coli* DH5α and JM101 and selected on ampicillin plates. It was found that no transformant was observed. The results of *E. coli* transformation was summarized in Table 3.1.

Subcloning of CGTase Gene from pCSBC5 to Modified Shuttle Vector pHV33 at *Eco*RI and *Hin*dIII Sites

This experiment was to subclone CGTase gene from pCSBC5 to a smaller shuttle vector made from pHV33. The cloning sites were at *Eco*RI and *Hin*dIII sites adjacent to chloramphenicol resistance gene (Fig.3.12). The pCSBC5 was cleaved with restriction endonucleases *Eco*RI and *Hin*dIII to separated CGTase gene containing fragment(5.2 kb) from the vector DNA (pUC18, 2.7 kb). The DNA fragment was separated by gel electrophoresis as shown in Fig.3.13 lane c. Then CGTase gene containing fragment (5.2 kb) was isolated from agarose gel by freeze-squeeze method for ligation with vector pHV33.

Table 3.1 Number of *E. coli* transformants obtained after ligation of transformation of restriction enzyme-digested plasmid DNAs

Plasmid DNAs treatment	No. of *E. coli* transformants*	
	DH5α	JM101
pBR322 (1 μg)	8.75×10^5	7.76×10^5
ligated *Hin*dIII-cleaved pBR322 (1 μg)	8.53×10^5	7.57×10^5
pHV33 (1 μg)	6.32×10^3	6.62×10^3
ligated *Bam*HI-cleaved pHV33	173	196
ligated *Hin*dIII-cleaved pSE411	220	217
ligated *Hin*dIII-cleaved pUC18	280	303
pC194 segment from *Hin*dIII-cleaved pHV33 ligated with *Hin*dIII-cleaved pCSBC8	0	0
pC194 segment from *Hin*dIII-cleaved pHV33 ligated with *Hin*dIII-cleaved pCSBC5	0	0
pC194 segment from *Hin*dIII-cleaved pHV33 ligated with *Hin*dIII-cleaved pSE411	0	0
pC194 segment from *Hin*dIII-cleaved pHV33 ligated with *Hin*dIII-cleaved pUC18	0	0

* No. of *E. coli* transformants per plasmid DNAs concentration of 250-300 ng in ligation reaction

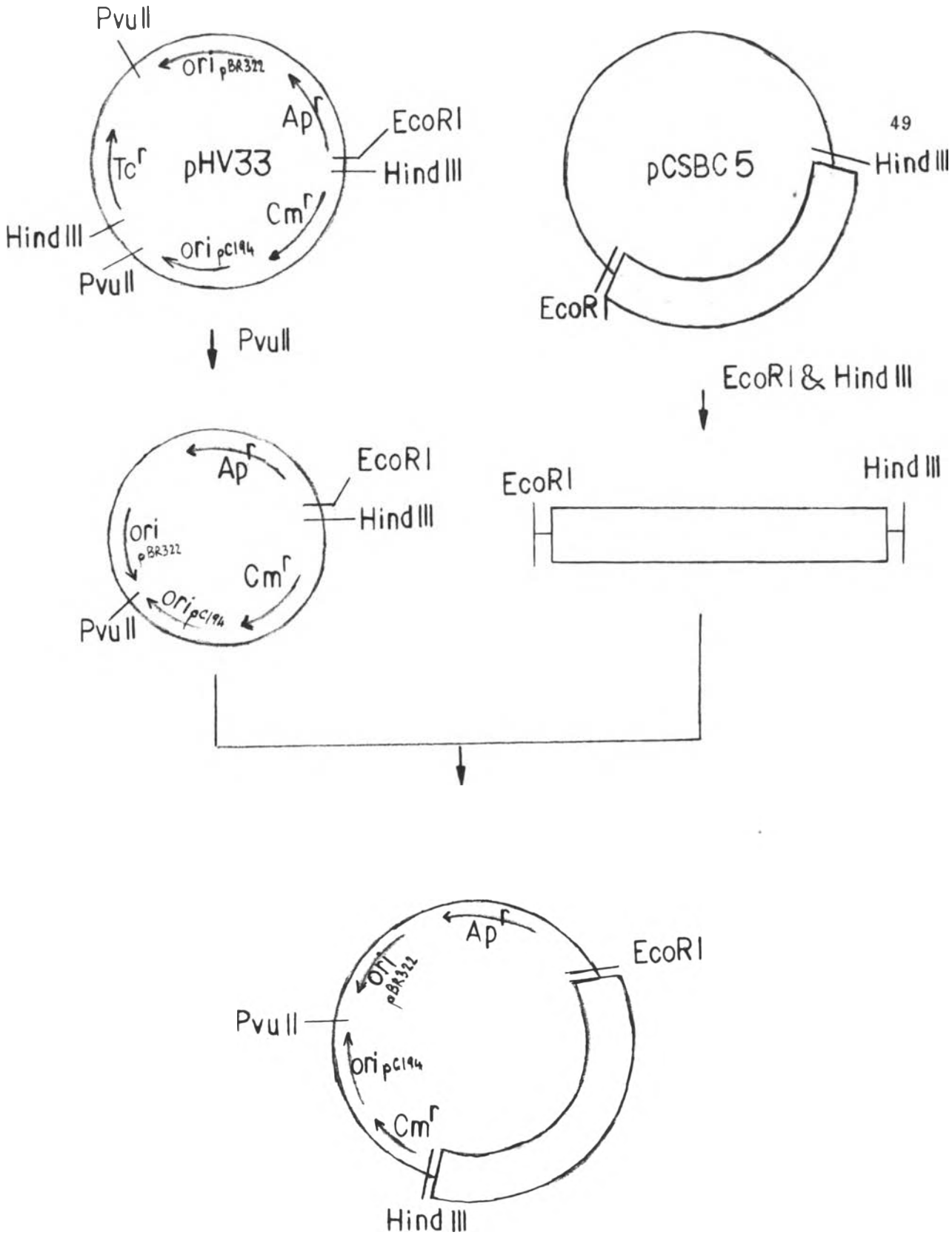


Figure 3.12 Subcloning of CGTase gene containing fragment from pCSBC5 to modified shuttle vector pHV33 at *Eco*RI and *Hind*III sites

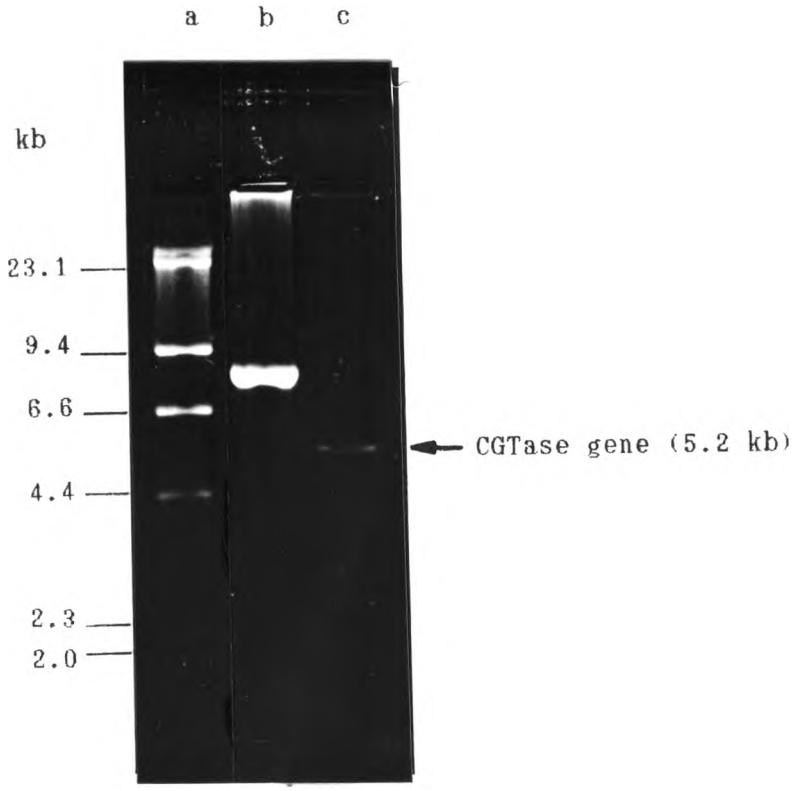


Figure 3.13 Electrophoretic analysis of pCSBC5 digested with restriction endonucleases *Hin*dIII and *Eco*RI order to demonstrate the separation of CGTase gene containing fragment

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC5 digested with *Hin*dIII

lane c : pCSBC5 digested with *Hin*dIII and *Eco*RI

There were two sites of *Hin*dIII in the vector pHV33, one site located on tetracycline resistance gene whereas the other site was near chloramphenicol resistance gene (Fig.3.12). This experiment was to subclone CGTase gene from *Hin*dIII and *Eco*RI-cleaved pCSBC5 to vector pHV33 at *Eco*RI and *Hin*dIII sites (located near chloramphenicol resistance gene). When vector pHV33 was cleaved with *Eco*RI and *Hin*dIII (located near chloramphenicol resistance gene), the linearized vector DNA was 6.8 kb. The distance between *Eco*RI and *Hin*dIII sites (located near chloramphenicol resistance gene) was only 29 bp. If the CGTase gene from *Eco*RI and *Hin*dIII cleaved pCSBC5 (5.2 kb) was subcloned to the linear form of vector pHV33 which was cleaved at *Eco*RI and *Hin*dIII sites (located near chloramphenicol resistance gene) (6.8 kb), the recombinant plasmid (about 12 kb) could not be transformed to the host cell *E. coli*. Therefore, the vector pHV33 would be shortened before cleaving with *Eco*RI and *Hin*dIII.

In order to remove one *Hin*dIII site adjacent to tetracycline resistance gene and to reduce the size of vector DNA, pHV33 was digested with *Pvu*II and religated. The tetracycline resistance gene was removed by this manipulation. Then the modified vector DNA was digested with *Eco*RI and *Hin*dIII and ligated with CGTase gene containing *Eco*RI, *Hin*dIII fragment as described above.

Fig.3.14 was the result of completely digested pHV33 with *Pvu*II (lane c), two DNA fragments of 4.5 kb and 2.3 kb were obtained. The large DNA fragment (4.5 kb) which carried the origin of replication of pBR322, pC194, ampicillin and chloramphenicol

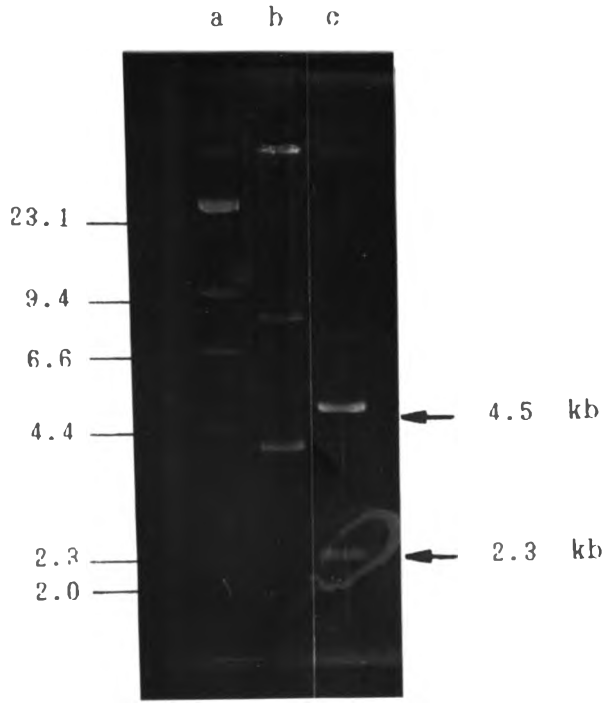


Figure 3.14 Electrophoretic analysis of *Pvu*II-cleaved pHV33

lane a : λ/*Hin*dIII (DNA marker)

lane b : pHV33

lane c : pHV33 digested with *Pvu*II

resistance genes was isolated from agarose gel by freeze-squeeze method. This large DNA fragment (4.5 kb) was ligated, transformed into *E. coli* JM101 and selected on ampicillin plates. The result of *E. coli* transformation was shown in Table 3.2

The result of Table 3.2 showed that no transformant which carried the target plasmid (4.5 kb) was observed. Since the vector DNA could not be prepared, subcloning of CGTase gene from pCSBC5 to the vector DNA could not be further performed.

Subcloning of CGTase Gene Containing Fragment from pCSBC5 to pUB110 at *Eco*RI and *Pvu*II Sites

This experiment was to subclone CGTase gene from pCSBC5 to pUB110 at *Eco*RI and *Pvu*II sites (Fig.3.15). The plasmid pUB110 was the vector for *Bacillus* host,and its restriction map was shown in Appendix VIII. The pUB110 was cleaved with restriction endonucleases *Eco*RI and *Pvu*II and analyzed by gel electrophoresis as shown in Fig.3.16 (lane c). Two DNA fragments were obtained. The large fragment (3.5 kb) which carried the origin of replication of pUB110 and kanamycin resistance gene was recovered from agarose gel by freeze-squeeze method for ligation with CGTase gene containing fragment from pCSBC5.

The CGTase gene containing DNA fragment from pCSBC5 was prepared by cleaving pCSBC5 with *Eco*RI and *Hin*cII. *Hin*cII and *Pvu*II digestions produced blunt-ended DNA, which can be ligated to each other.

Table 3.2 Number of *E. coli* transformants obtained after ligation and transformation of plasmid DNAs

Plasmid DNAs	No. of *E. coli* transformants*
ligated *Pvu*II-cleaved pBR322	123
pHV33 (1 μg)	6.62×10^3
ligated *Bam*HI-cleaved pHV33	196
ligated 4.5 kb DNA fragment from *Pvu*II-cleaved pHV33	0

* No. of *E. coli* transformants per plasmid DNAs concentration of 250-300 ng in ligation reaction

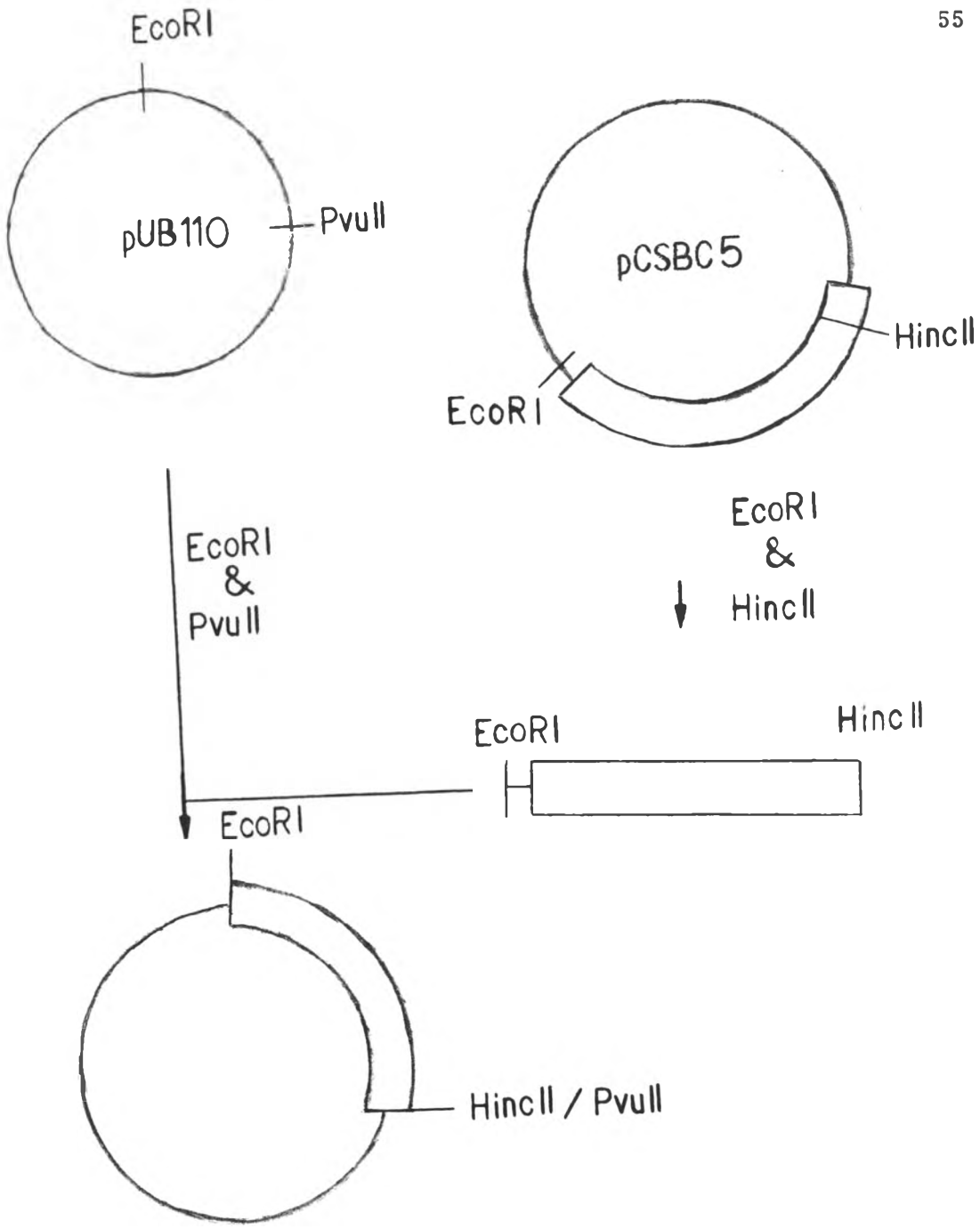


Figure 3.15 Subcloning of CGTase gene containing fragment from pCSBC5 to pUB110 at *Eco*RI and *Pvu*II sites

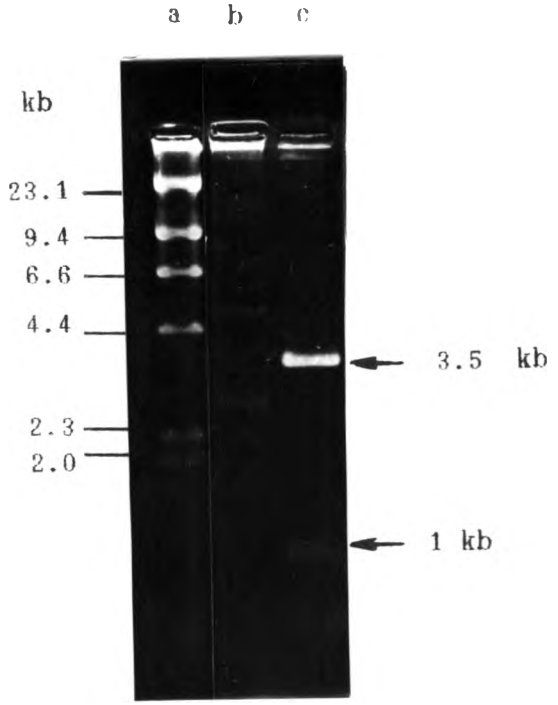


Figure 3.16 Electrophoretic analysis of vector pUB110 digested with restriction endonucleases *Eco*RI and *Pvu*II

lane a : λ/*Hin*dIII (DNA marker)

lane b : pUB110

lane c : pUB110 digested with *Eco*RI and *Pvu*II

The digested pCSBC5 was analyzed with gel electrophoresis as shown in Fig.3.17. The result in Fig.3.17 showed that plasmid pCSBC5 was cleaved to a single linear DNA fragment of 7.9 kb by *Eco*RI (lane c). The double digestion with *Eco*RI and *Hin*cII (lane d), yielded two DNA fragments of 3.9 kb and 2.7 kb.

In contrast to the result obtained, the expected DNA fragments from the digestion of pCSBC5 with *Eco*RI and *Hin*cII should be of 4.7 kb (CGTase gene containing fragment) and 3.2 kb. The result in Fig.3.17 lane d showed that two DNA fragments of about 3.9 kb and 2.7 kb which were not target DNA fragments, were obtained. The result indicated that there may be other *Hin*cII sites on CGTase gene of pCSBC5. Therefore, the complete CGTase gene could not be separated from pCSBC5, and the subcloning of CGTase gene from pCSBC5 to vector plasmid pUB110 could not be done successfully.

Detection and Isolation of CGTase Gene by DNA-DNA Hybridization to Chromosomal DNA of *Bacillus* sp. A11 Using pCSBC5 as a Probe

The results described above showed that the subcloning of CGTase gene from pCSBC5 and pCSBC8 to the shuttle vector pHV33 and *Bacillus* plasmid vector pUB110 could not be done successfully. Moreover, both pCSBC5 and pCSBC8 had lost their CGTase activity as detected by iodine test and CD-TCE complex formation. In order to construct a new stable recombinant clone for CGTase production, an experiment was set up to isolate the CGTase gene from *Bacillus* sp. A11

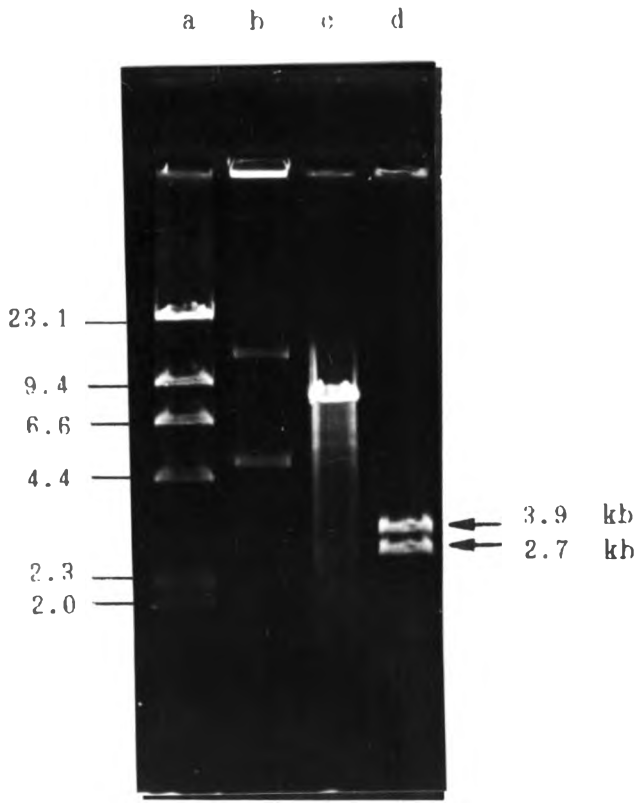


Figure 3.17 Electrophoretic analysis of pCSBC5 digested with restriction endonucleases *Eco*RI and *Hin*cII

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC5

lane c : pCSBC5 digested with *Eco*RI

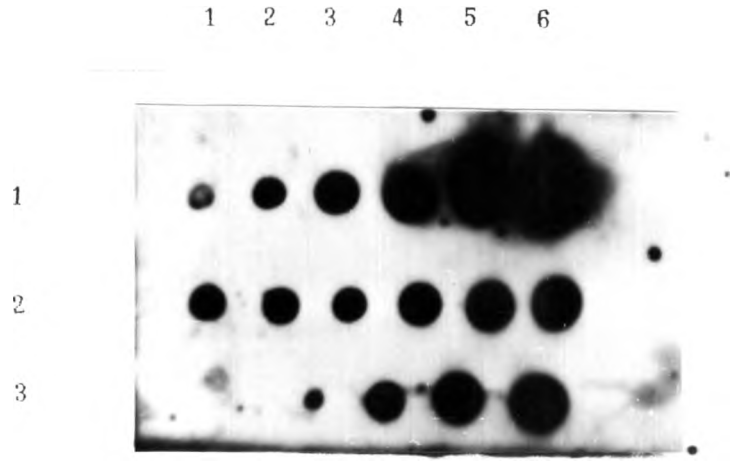
lane d : pCSBC5 digested with *Eco*RI and *Hin*cII

by using pCSBC5 as DNA probe.

Since the plasmid pCSBC5 was a plasmid vector pUC18 carrying CGTase gene from *Bacillus* sp. A11, it had to prove first that pUC18 would not hybridize with chromosomal DNA from *Bacillus* sp. A11 before the pCSBC5 could be used as DNA probe. The plasmids pCSBC5 and vector pUC18 were labeled with the Genius nonradioactive labeling system via random primed DNA labeling with DIG-dUTP. The yield of nonradioactive labeled pCSBC5 and pUC18 probes were estimated as shown in Fig.3.18.

The result of Fig.3.19 row 1 demonstrated hybridization between plasmid pACYC184 (no.3), pSA30 (no.4) and labeled pCSBC5 (A), pUC18 (B) probe. Fig.3.19 A row 2 showed some hybridization the labeled pCSBC5 probe and the chromosal DNA of *Bacillus* sp. A11. The result of Fig.3.19 B row 2 showed that the chromosomal DNA of *Bacillus* sp. A11 did not hybridize with labeled pUC18 probe. It indicated that the plasmid vector pUC18 was not homology with the chromosomal DNA of *Bacillus* sp. A11. Therefore, the plasmid pCSBC5 could be used as DNA probe for hybridization with the chromosomal DNA of *Bacillus* sp. A11 in order to detect the chromosomal counterpart of CGTase gene. The chromosomal DNA of *Bacillus* sp. A11 was completely digested with restriction endonucleases, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*II or partially digested with restriction endonuclease *Sau*3AI, analyzed by agarose gel electrophoresis, Southern blot transferred and hybridized with labeled pCSBC5 probe. The results were shown in Fig.3.20.

Figure 3.18 Estimation of DNA concentration of nonradioactive DIG-labeled probes by chemiluminescent detection



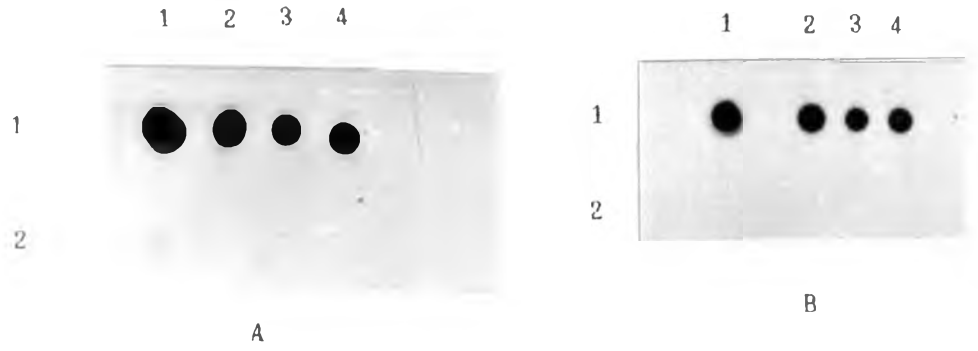
One microliter of each ten-fold serial dilution of Digoxigenated DNA probes was spotted onto the nylon membrane. The signal intensities were visually compared with the labeled control DNA from the kit. The labeled control DNA concentration from 0.01 pg/μl to 1 ng/μl

row 1 no.1-6 : the labeled control DNA 0.01 pg/μl to 1 ng/μl

row 2 no.1-6 : the labeled pUC18 probe

row 3 no.1-6 : the labeled pCSBC5 probe

Figure 3.19 Chemiluminescent dot blot hybridization of chromosomal DNA of *Bacillus* sp. A11 with DIG-labeled probes



1 μg of chromosomal DNA from *Bacillus* sp. A11 was diluted to obtain two-fold series of dilution, spotted onto a membrane, fixed and hybridized with pCSBC5 probe (A) and pUC18 probe (B) (about 5 ng/ml). The hybridization was performed at 65°C for at least 12 h and detected by chemiluminescent detection for 1 h.

row 1 no.1-4 : 1 μg plasmid pCSBC5, 1 μg plasmid pUC18, 1 μg plasmid pACYC184, 1 μg plasmid pSA30 (recombinant plasmid constructed from cloning *nif* HDYK of *Klebsiella pneumoneae* at *Eco*RI site of plasmid vector pACYC184)

row 2 no.1-4 : chromosomal DNA of *Bacillus* sp. A11 1 μg, 0.5 μg, 0.25 μg, 0.125 μg

Fig.3.20 B showed that there were intense hybridized signals with the DNAs, after digestion with *Bam*HI (lane b), *Eco*RI (lane c), *Hin*dIII (lane d), or *Pst*I (lane e) of approximately 2.5 kb. The hybridized signals of 2.5 kb should be CGTase gene containing fragment in chromosomal DNA of *Bacillus* sp. A11 after digestion, because they were homology with CGTase gene containing fragment in the labeled pCSBC5 probe. Therefore, about 2.5-4.4 kb DNA fragments of *Hin*dIII-digested *Bacillus* sp. A11 chromosomal DNA were chosen for the cloning of the hybridized fragment.

Cloning of CGTase Gene from *Bacillus* sp. A11

The chromosomal DNA of *Bacillus* sp. A11 was digested with *Hin*dIII, and DNA fragments size of about 2.5-4.4 kb were recovered from agarose gel using Geneclean Kit. Then the DNA fragments were cloned to vector pUC18 at *Hin*dIII site and transformed into competent cells *E. coli* JM101. The transformants were selected on L-agar plate containing 50 µg/ml of ampicillin, 60 µg/ml of IPTG (isopropylthio-β-D-galactoside) and 100 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) as shown in Table 3.3. Only white colonies were selected for the detection of CGTase gene.

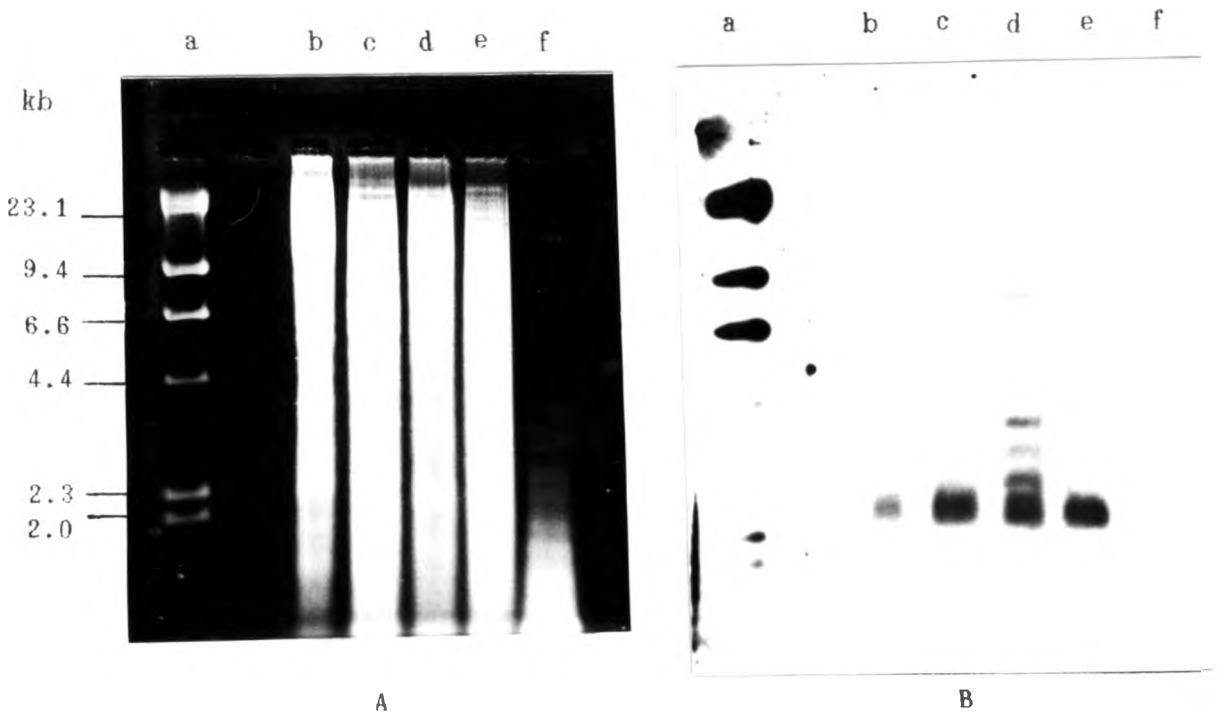


Figure 3.20 Electrophoretic analysis (A) and Southern blot hybridization of (A) with nonradioactive labeled pCSBC5 probe (B)

lane a : λ/*Hin*dIII (DNA marker)

lane b : chromosomal DNA of *Bacillus* sp. A11 completely digested with *Bam*HI

lane c : chromosomal DNA of *Bacillus* sp. A11 completely digested with *Eco*RI

lane d : chromosomal DNA of *Bacillus* sp. A11 completely digested with *Hin*dIII

lane e : chromosomal DNA of *Bacillus* sp. A11 completely digested with *Pst*I

lane f : chromosomal DNA of *Bacillus* sp. A11 partially digested with *Sau*3AI for 60 min

Table 3.3 Number of transformants from the cloning of chromosomal DNA of *Bacillus* sp. A11, digested with *Hin*dIII, into plasmid vector pUC18 at *Hin*dIII site.

Size of DNA fragments recovery (kb)	No. of transformants	
	blue colonies	white colonies
2.5-4.4	807	26

Detection of Recombinant Plasmids that Carried CGTase Gene

1. Dextrinizing activity test (I_2 method)

All white colonies were replicated to L-agar plates containing 50 μg/ml of ampicillin and 1% (w/v) of soluble starch. Dextrinizing activity of transformants was assayed by iodine test. It appeared that only one white colony, namely no.17, showed clear zone around the colony. Cloning experiments as mentioned above have been repeated several times in order to find more positive clones. More than 1,000 white colonies derived from the cloning into pUC18 of DNA fragments size 2.5-4.4 kb from *Hin*dIII-digested chromosomal DNA were tested negative for halo formation.

It was later found out that the transformant no.17 could not produce clear zone around the colony upon iodine test, after subcultured. It indicated that the transformant no.17 was not a stable clone.

Moreover, recombinant plasmid was extracted from the transformant no.17 by minipreparation, digested with *Hin*dIII and analyzed by agarose gel electrophoresis (data not shown). Two DNA fragments of 3.54 kb and 2.6 kb were obtained. The DNA fragment of 3.54 kb probably contained CGTase gene from *Bacillus* sp. A11, whereas the DNA fragment of 2.6 kb was the linear form of vector pUC18.

2. DNA-DNA hybridization with labeled CGTase grne (5.2 kb) containing probe from pCSBC5

Since, the recombinant plasmids, pCSBC5 and transformant no.17 carried pUC18 vector DNA (Appendix VII). Thus, the DNA insert containing CGTase gene was prepared from pCSBC5 by digesting the pCSBC5 with restriction endonuclease *Pst*I (Appendix VI) to use as DNA probe. The CGTase gene containing fragment was separated by agarose gel electrophoresis, recovered from agarose gel using Geneclean Kit and then labeled with the Genius nonradioactive labeling system. The labeled CGTase gene (5.2 kb) probe was hybridized with *Hin*dIII-digested recombinant plasmid extracted from transformant no.17 (data not shown). It appeared that there were hybridized signals with DNA insert DNA (3.54 kb) and also at the band of linear form of vector pUC18 (2.6 kb). It indicated that part of the multiple cloning sites of vector pUC18 in the labeled CGTase gene (5.2 kb) probe could hybridize with linear form of vector pUC18 fragment after the digestion of the recombinant plasmid no.17 with *Hin*dIII. In order to avoid this, part of the CGTase gene containing fragment from pCSBC5 was then used as DNA probe.

3. DNA-DNA hybridization with labeled CGTase gene fragment (1.7 kb) probe from pCSBC5

Part of the CGTase gene containing fragment was prepared from pCSBC5 by digestion of pCSBC5 with *Pvu*II and *Sal*I (Appendix VI) and isolation of the fragment using agarose gel electrophoresis. The

DNA fragment (1.7 kb) was purified from agarose gel by Geneclean Kit and then labeled with Genius nonradioactive labeling system.

Since the transformant no.17 was the only clone which had ever been shown to have dextrinizing activity by iodine test, the recombinant plasmid no.17 was used in the detection of CGTase gene. The *Hin*dIII-digested plasmid from clone no.17, and the *Pst*I-cleaved pCSBC5 CGTase gene containing fragment (5.2 kb) were then subjected to agarose gel electrophoresis, Southern blot transferred and hybridized with the labeled CGTase gene fragment (1.7 kb) probe as shown in Fig.3.21. The result showed that there was hybridization signal with DNA fragment of 3.54 kb from recombinant plasmid clone no.17 (lane b). It indicated that the recombinant plasmid no.17 carried the CGTase gene from *Bacillus* sp. A11.

4. DNA-DNA hybridization with labeled CGTase gene fragment (3 kb) probe

Since the CGTase gene fragment (1.7 kb) probe was just part of the CGTase gene containing fragment (5.2 kb) from pCSBC5, the other part of the insert from pCSBC5 was used as DNA probe in order to verify the recombinant plasmid no.17 whether it contained a similar DNA fragment.

The CGTase gene containing fragment (3 kb) was prepared from pCSBC5 by digestion of pCSBC5 with *Pst*I and *Pvu*II (Appendix VI) and subjected to agarose gel electrophoresis. The insert fragment (3 kb)

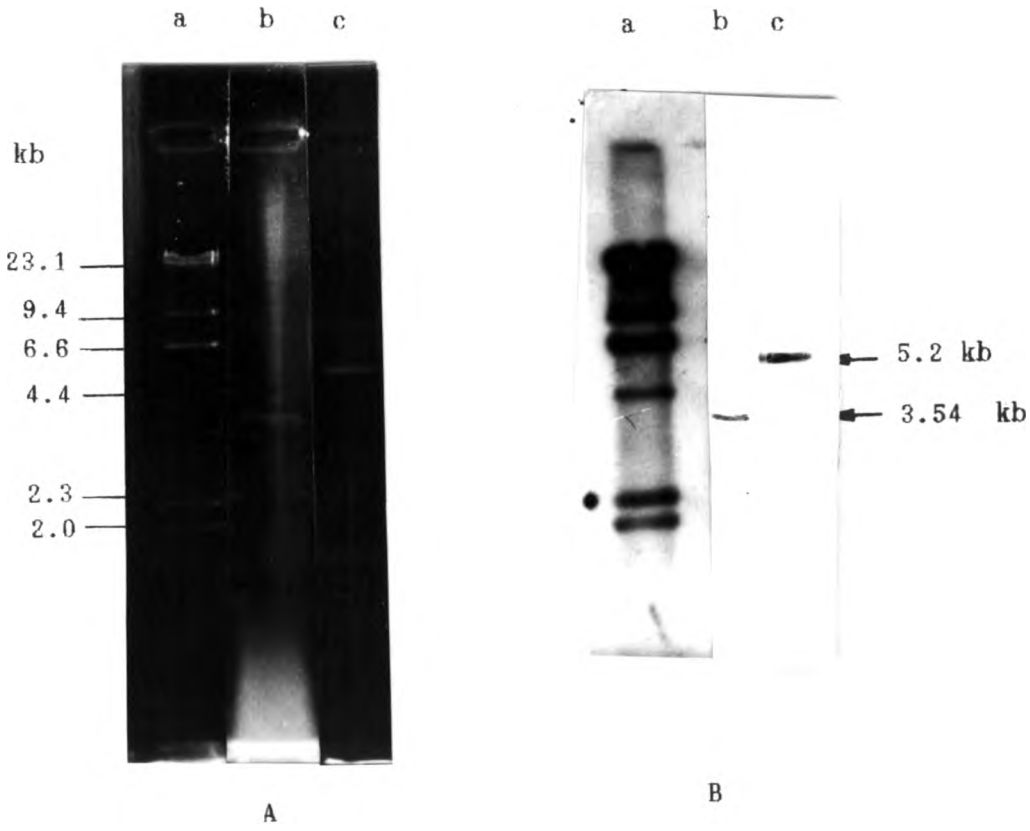


Figure 3.21 Electrophoretic analysis (A) and Southern blot hybridization of (B) with nonradioactive labeled 1.7 kb CGTase gene fragment probe (B)

lane a : λ/*Hin*dIII (DNA marker)

lane b : *Hin*dIII-cleaved recombinant plasmid no.17

lane c : *Pst*I-cleaved pCSBC5

(5.2 kb CGTase gene containing fragment)

was purified from agarose gel by Geneclean Kit and then labeled with Genius nonradioactive labeling system.

The plasmid pUC18 which was digested with *Hin*dIII, the plasmid pCSBC5 which was digested with *Pst*I, the plasmid pUB110 which was digested with *Eco*RI, and the 3.54 kb insert fragment from *Hin*dIII-cleaved recombinant plasmid no.17 were analyzed by agarose gel electrophoresis, Southern blot transferred and hybridized with the labeled 3 kb CGTase gene fragment probe. The results were shown in Fig.3.22.

The result in Fig.3.22 showed that hybridization signal was observed with 3.54 kb insert fragment from *Hin*dIII-cleaved recombinant plasmid no.17 (lane d), indicating that the recombinant plasmid no.17 should carry the CGTase gene from *Bacillus* sp. A11. However, hybridization signal was also detected with the linear form of *Hin*dIII-digested vector pUC18 (2.6 kb) in lane c. This was because there was part of the multiple cloning sites (from *Pst*I to *Bam*HI sites) of vector pUC18 in the labeled 3 kb CGTase gene fragment.

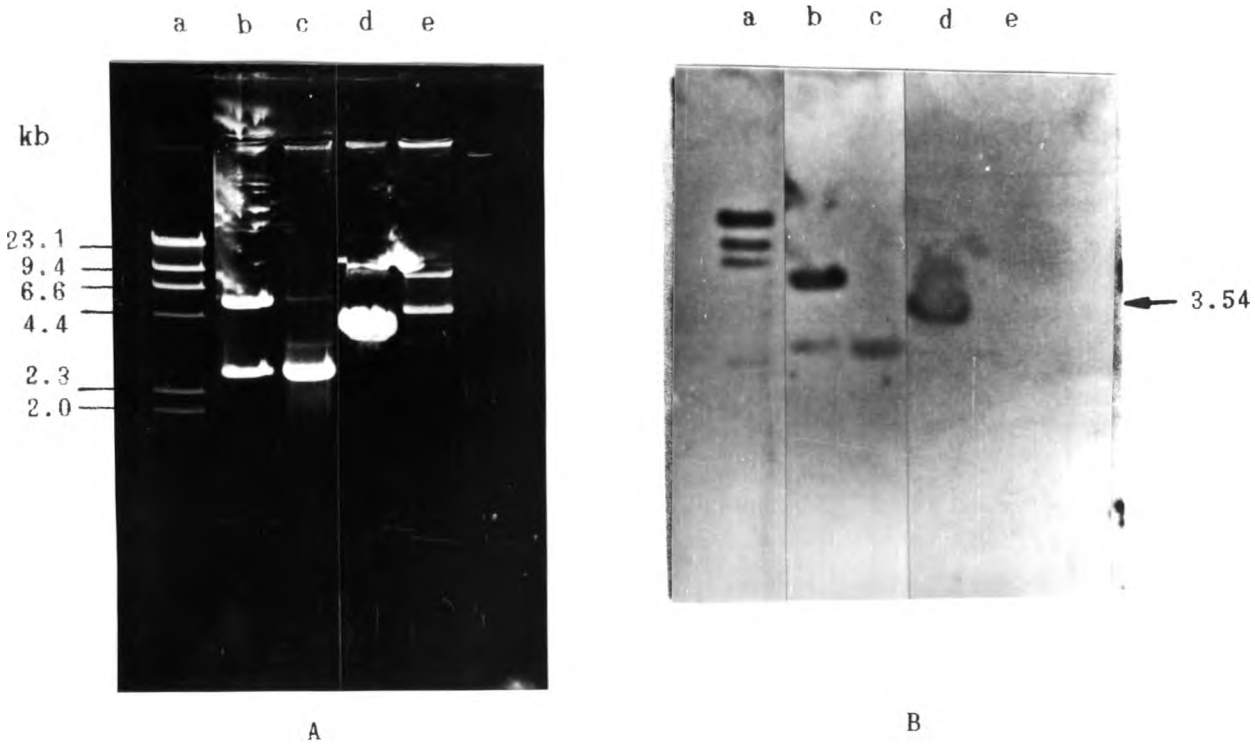


Figure 3.22 Electrophoretic analysis (A) and Southern blot hybridization of (A) with nonradioactive labeled 3 kb CGTase gene fragment probe (B)

lane a : λ/*Hin*dIII (DNA marker)

lane b : pCSBC5 digested with *Pst*I

lane c : pUC18 digested with *Hin*dIII

lane d : the recombinant plasmid no.17 digested with *Hin*dIII

lane e : pUB110 digested with *Eco*RI