CHAPTER III

RESULTS

3.1. Detection and preparation of plasmid DNA.

Since free-living Klebsiella pneumoniae M5a1 posseses no plasmid and all the nif regulon is in the chromosome, the first question is to clarify whether associative Klebsiella strain R15 and R17 contain any plasmid. In order to solve this question three conventional methods generally used for the determination of plasmid in bacteria were performed with strain R15 and R17, as well as E. coli strains habouring plasmids of different sizes namely pBR322 (4.36 kb), pRD1 (86 kb) and pSA30 (10.3 kb). The associative K. oxytoca NG13 was also used as control for plasmidless strain.

3.1.1. Plasmid detection by modified Eckhardt (1978) method.

By this method, the spheroplasts from 1-2 colonies of bacteria are gently lysed by SDS in gel slots and the chromosomal and plasmid DNA are then separated by electrophoresis. So that, the bulk of the chromosomal DNA are intact and mostly excluded from the gel under the condition used. Only plasmid DNA should migrate through the pore of agarose gel as covalently closed circular form (ccc).

Figure 3.1 shows that both associative Klebsiella strain R15 and R17 contain no distinct plasmid DNA band, whereas the E. coli strains show distinct plasmid bands close to 4.4 kb (pBR322), 10.3 kb (pSA30) and smeared band above chromosomal DNA (pRD1, 86 kb) respectively.

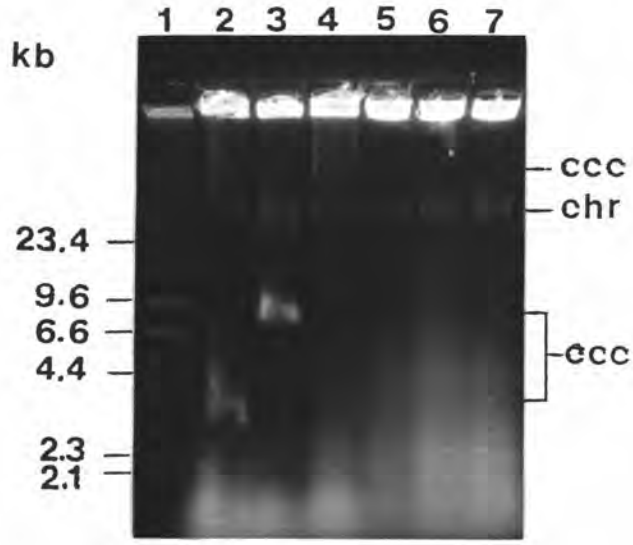


Figure 3.1. Gel electrophoresis of lysate from E. coli strains harbouring plasmid and associative Klebsiella spp. by modified Eckhardt's method.

After lysozyme treatment, the spheroplasts were lysed by migrating SDS and then chromosomal DNA (chr) and covalently closed circular plasmid (ccc) were separated in agarose gel electrophoresis.

lane 1 : λ/HindIII

lane 2 : E. coli (pBR322, 4.36 kb)

lane 3 : E. coli (pSA30, 10.3 kb)

lane 4 : E. coli (pRD1, 86 kb)

lane 5 : K. oxytoca NG13

lane 6 : K. R15

lane 7 : K. R17

3.1.2. Plasmid detection by Kado and Liu (1981) method.

In this procedure, bacterial cells are lysed in alkali-detergent solution and followed by high temperature treatment to eliminate the "feathery" effect and chromosomal DNA, and at the same time to enhance plasmid recovery.

Figure 3.2.a shows that various sizes of plasmids in E. coli strains were clearly detected in multiple forms especially after heat treatment at 55^0c for 30 or 60 min (lane 3, 4, 6, 7, 9 and 10). In case of associative Klebsiella strain R15 and R17, Figure 3.2.b obtained from similar preparation method even with treatment at 65^0c indicate the absence of plasmid.

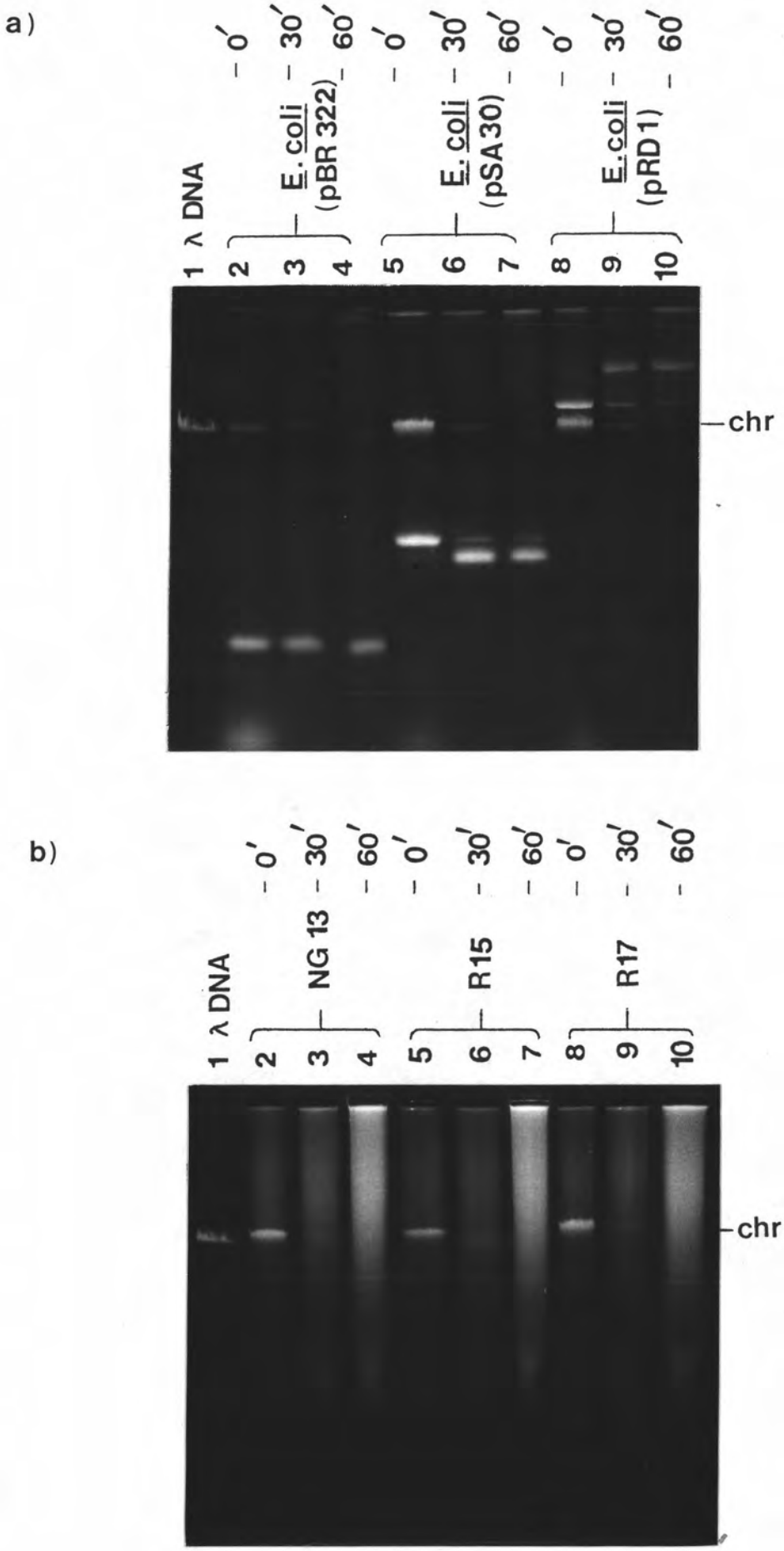
3.1.3. Plasmid detection by rapid alkaline extraction (Birnboim and Doly, 1979).

In this procedure, denatured chromosomal DNAs are mostly precipitated with a high concentration of salt (about 1M sodium acetate, pH 4.8) and left smaller plasmid DNA in the soluble fraction. The bulk of cellular RNAs and proteins are also precipitated under these conditions.

Figure 3.3. shows that none of the plasmid band has been detected in the associative Klebsiella strain R15 and R17 where as pBR322, and pSA30 are present. This result confirms the absence of plasmid in strains R15 and R17 as well as the other two rapid methods described previously.

3.1.4. Preparation of plasmid for Southern hybridization.

Since plasmid obtained from rapid alkaline extraction may be contaminated with RNAs and sometimes with high molecular weight DNA, the obtained plasmids:pBR322, pSA30 and pAM51 were further purified by


a)
1 λ DNA
2 – 0′
3 – E. coli (pBR 322) – 30′
4 – 60′
5 – 0′
6 – E. coli (pSA30) – 30′
7 – 60′
8 – 0′
9 – E. coli (pRD1) – 30′
10 – 60′
chr
b)
1 λ DNA
2 – 0′
3 – NG 13 – 30′
4 – 60′
5 – 0′
6 – R15 – 30′
7 – 60′
8 – 0′
9 – R17 – 30′
10 – 60′
chr

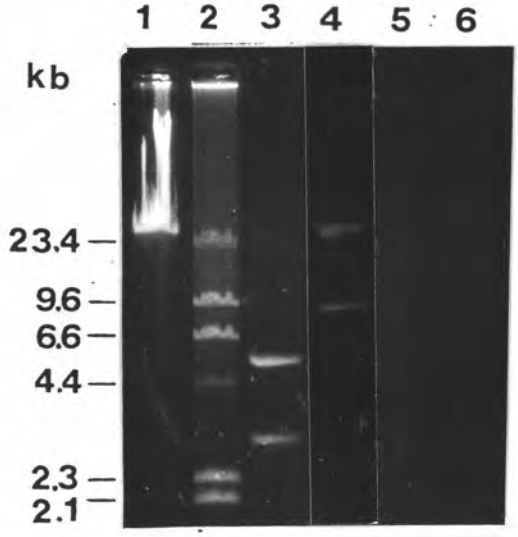


Figure 3.3. Agarose gel electrophoresis of plasmid DNA prepared by rapid alkaline extraction.

After lysozyme treatment, cells were lysed in alkali-SDS. Upon neutralization, denatured chromosomal DNA was precipitated with 3 M sodium acetate, pH 4.8 whereas plasmid DNA remained in the soluble fraction. After centrifugation, plasmid DNA was recovered from solution by ethanol precipitation and redissolved for agarose gel electrophoresis.

lane 1 : λ DNA, 49 kb

lane 2 : λ/HindIII

lane 3 : pBR322, 4.36 kb

lane 4 : pSA30, 10.3 kb

lane 5 : K. R15, no plasmid detected

lane 6 : K. R17, no plasmid detected

isopycnic centrifugation in cesium chloride gradient containing ethidium bromide. The purity of plasmids were evident by the ratio of absorbance at 260 and 280 nm (A_{260nm}/A_{280nm}) of 2.0 and the plasmid profiles displaying multiple forms (ccc and relaxed) were shown in Figure 3.4. These purified plasmid DNA were used as DNA probes in Southern hybridization experiment.

3.2. Preparation of chromosomal DNA.

High molecular weight chromosomal DNAs with high purity were obtained after extraction by Rodriguez and Tsit (1983) as shown in Figure 3.5 and the ratio of A_{260nm}/A_{280nm} were in the range of 1.8-1.9.

3.3. Comparision of Restriction Fragment Length Polymorphism (RFLP) of free-living and associative Klebsiella strains.

In this study, some type II restriction endonucleases were selected namely; BamHI, BglII, EcoRI, HindIII, PstI, SalI, SmaI and XhoI because their recognition sequences of six nucleotides are known to be in the genes of interest in K. pneumoniae.

Complete cutting of 1 µg chromosomal DNA were observed after digestion at 37°C for 3 h either by 10 units or 20 units of each restriction enzyme as shown in Figure 3.6. Therefore, 30 units of each enzyme were used for cutting 3 µg chromosomal DNA in Southern blot transfer. The digested fragments of chromosomal DNA were analyzed on 12 x 15 x 0.5 cm agarose gel. Figure 3.7 shows that the RFLP patterns of digested DNA from Klebsiella spp. of all these enzymes indicate the different patterns between free-living K. pneumoniae M5a1 and the associative Klebsiella strains : K. oxytoca NG13, K. R15 and K. R17, and among these 3 associative strains, similar RFLP patterns have been observed.

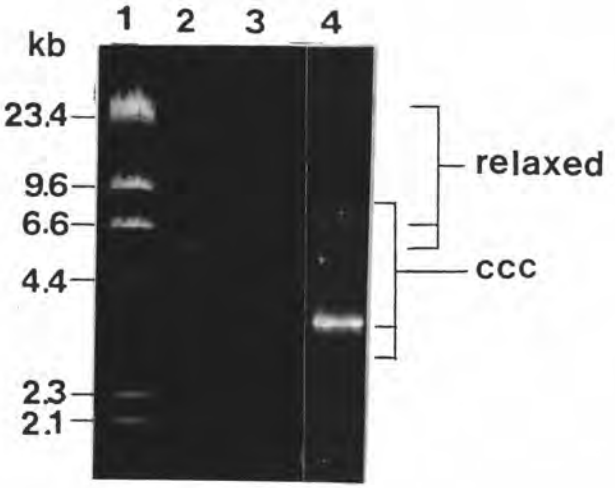


Figure 3.4. Gel electrophoresis of pBR322, pSA30 and pAM51 after purification by isopycnic centrifugation.

After rapid alkaline extraction, the plasmids were further purified by isopycnic centrifugation in cesium chloride gradient containing ethidium bromide. After removing of cesium chloride and ethidium bromide, the purified plasmids (ccc and relaxed form) were analyzed by agarose gel electrophoresis.

lane 1 : λ/HindIII

lane 2 : pBR322, 4.36 kb

lane 3 : pSA30, 10.3 kb

lane 4 : pAM51, 5.1 kb

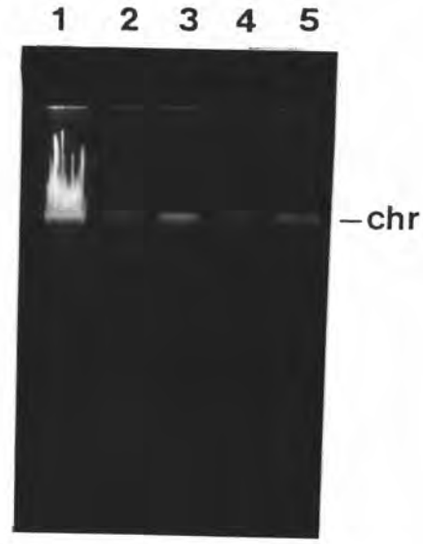


Figure 3.5. The chromosomal DNA of Klebsiella spp. extracted by method of Rodriquez and Tsit (1983).

One hundred nanograms of extracted chromosomal DNA (chr) was loaded in each well of 0.7% agarose gel and electrophoresed in Tris-borate buffer, pH 8.3 at 80 volts for 3 h.

lane 1 : λ DNA

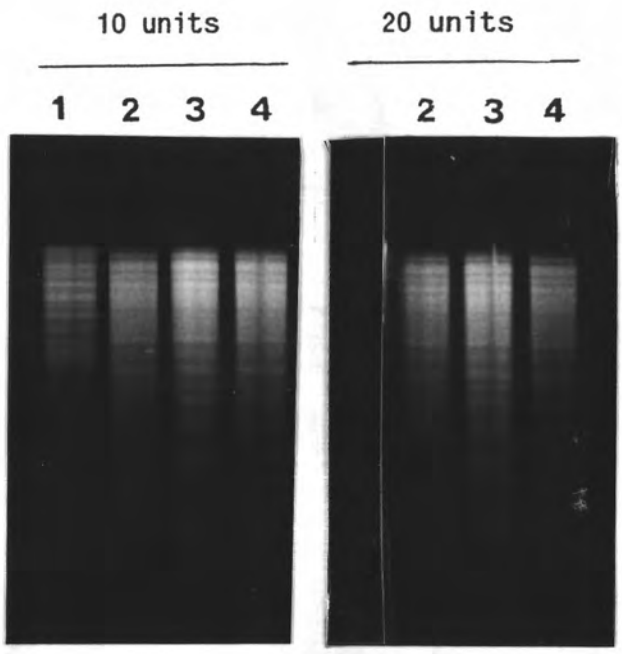
lane 2 : K. pnemoniae M5a1

lane 3 : K. oxytoca NG13

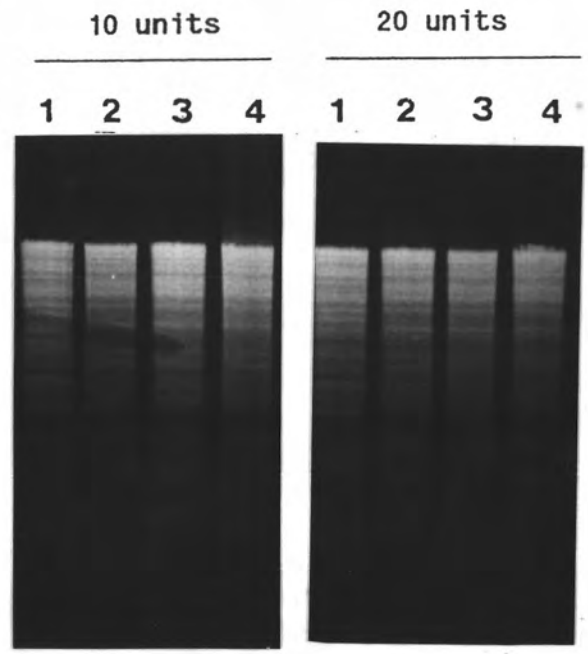
lane 4 : K. R15

lane 5 : K. R17

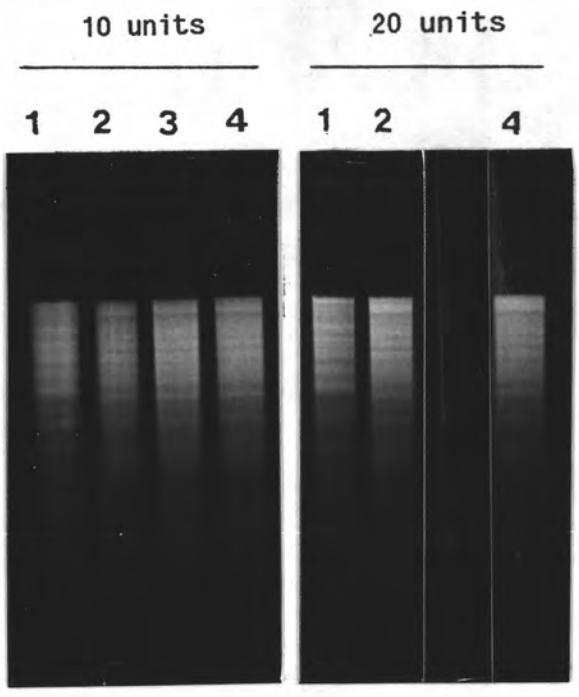
a) BamHI



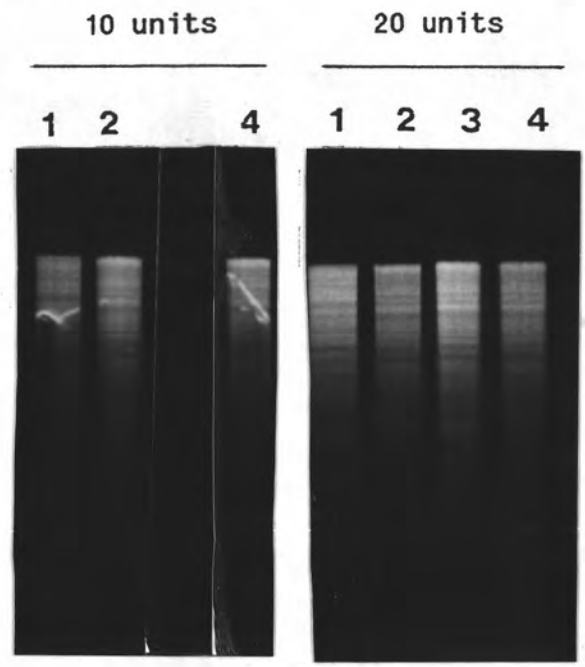
b) BglII



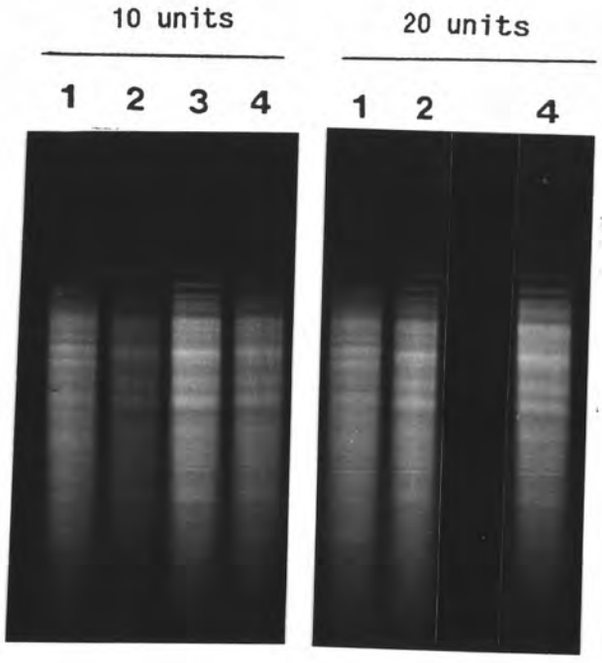
c) EcoRI



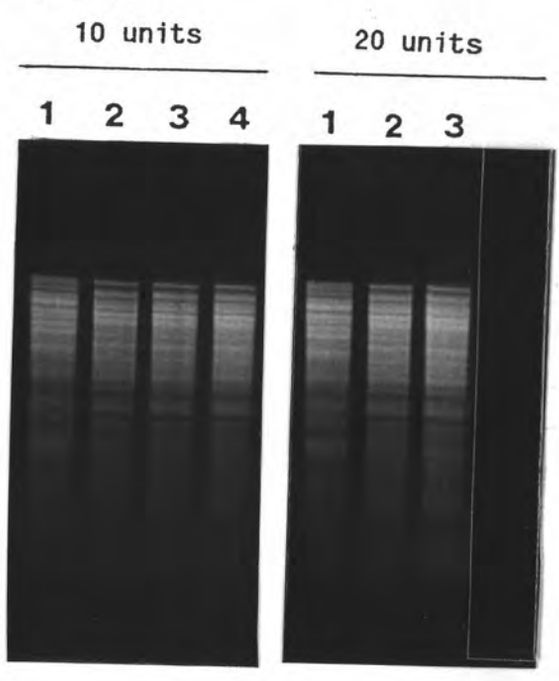
d) HindIII



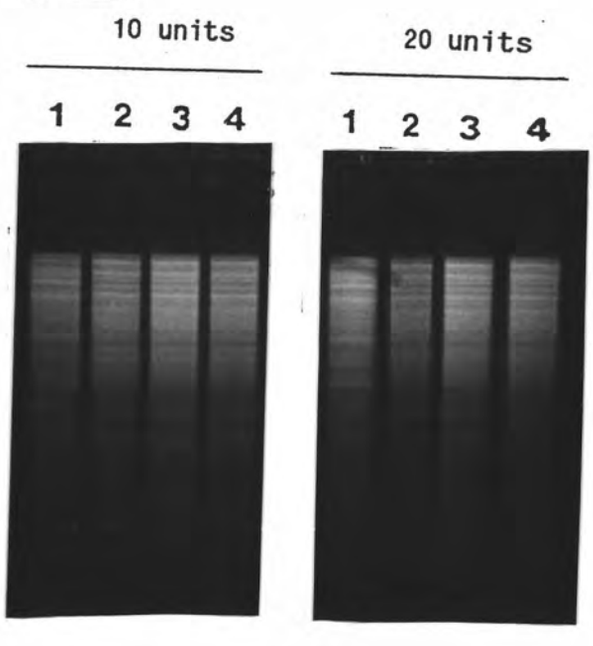
e) PstI



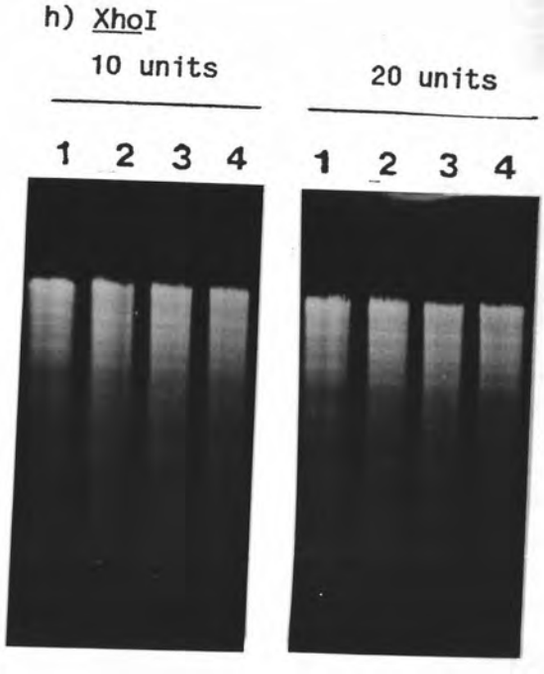
f) SalI

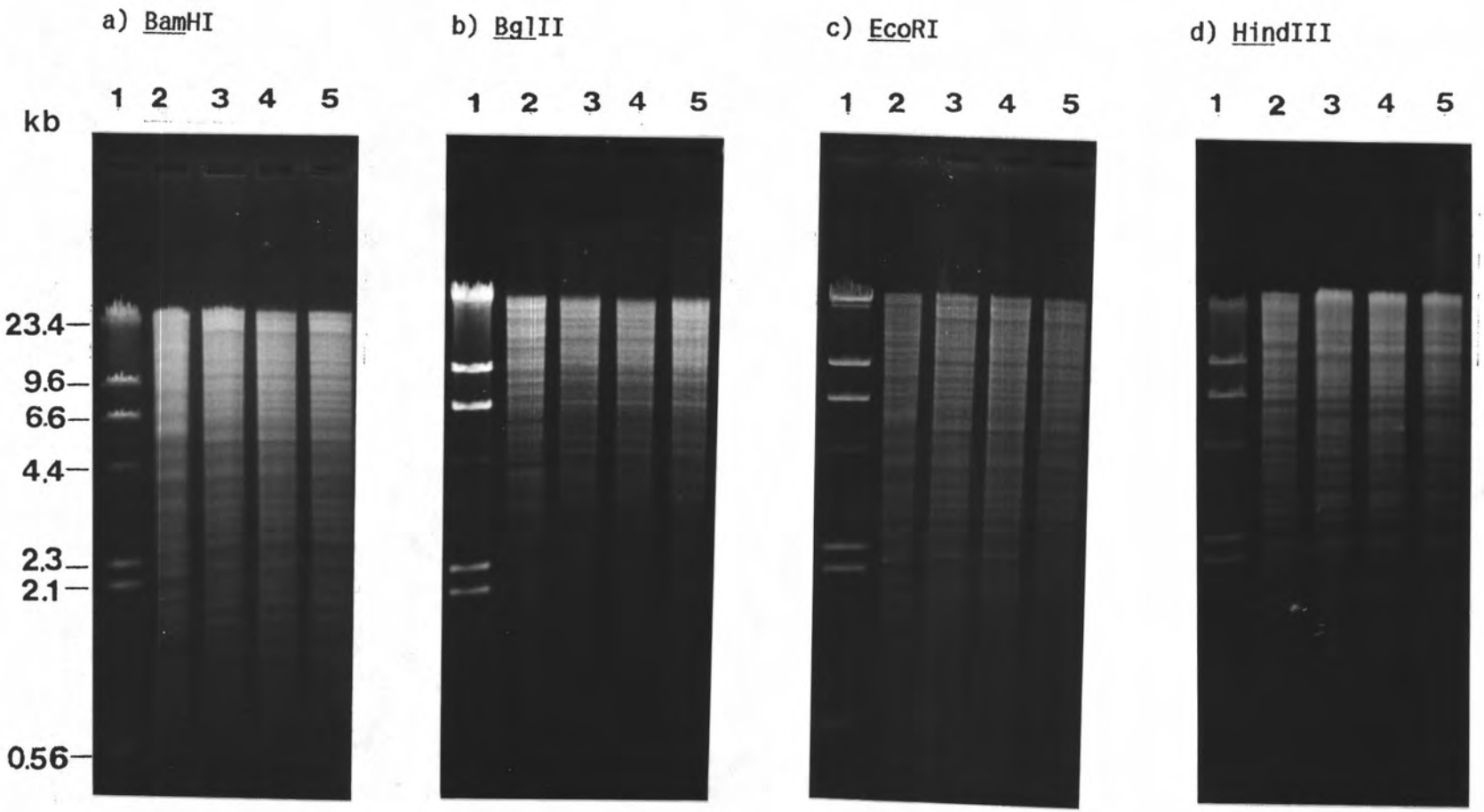


g) SmaI



h) XhoI




a) BamHI
b) BglII
c) EcoRI
d) HindIII
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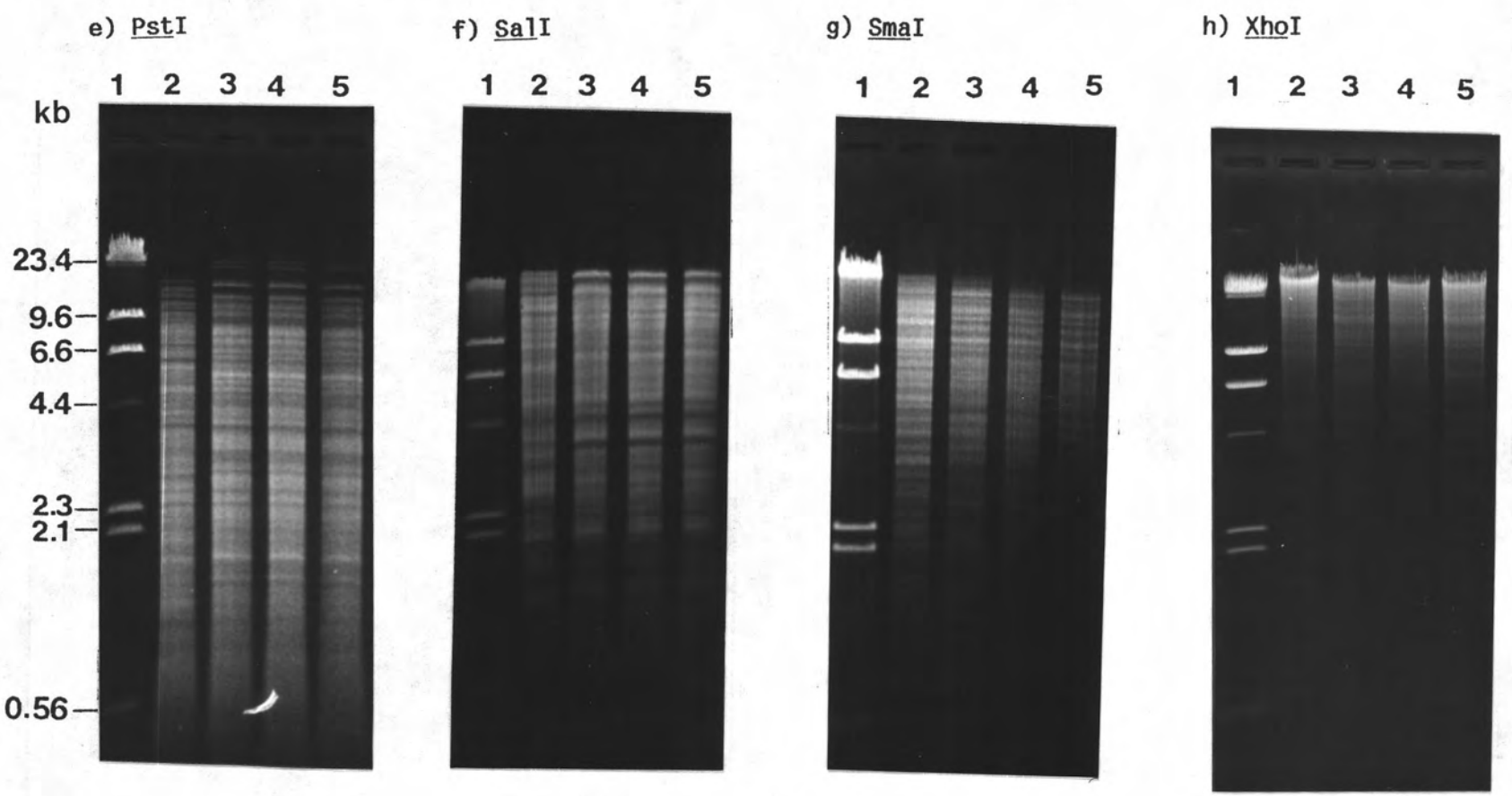
kb
23.4
9.6
6.6
4.4
2.3
2.1
0.56


e) PstI
f) SalI
g) SmaI
h) XhoI
1 2 3 4 5
kb
23.4
9.6
6.6
4.4
2.3
2.1
0.56

3.4. Comparison of RFLP by nif structural genes.

To find out whether the DNA homology between nif genes of K. pneumoniae M5a1 and those of associative Klebsiella strains exists, the pSA30 plasmid containing nif HDK structural genes of K. pneumoniae (Figure 1.1 b) was purified and digested with EcoRI, BamHI and HindIII to confirm for the restriction patterns (Figure 3.8). The pSA30 plasmid was labelled with [α-^{32}P] dCTP by nick translation reaction. From the kinetic study of nick translation reaction shown in Figure 3.9, the maximum incorporation of [α-^{32}P] dCTP into pSA30 is 72% at 60 min. The specific activity of ^{32}P-pSA30 probe prepared under this condition is 8.34×10^8 cpm/µg DNA. Southern hybridization of this labelled probe with chromosomal DNA fragments digested with each enzyme (Figure 3.10) shows the similar RFLP patterns of BamHI, EcoRI, HindIII, SalI and XhoI cutting between the free-living K. pneumoniae and the associative Klebsiella strains : K. oxytoca NG13, K. R15 and K. R17. Different patterns have been observed with BglII, PstI and SmaI cutting (Figure 3.11). The sizes of these hybrid bands have been summarized in Table 3.1. These results indicates that the restriction patterns of the nif structural genes region of these associative Klebsiella strains are very similar to K. pneumoniae M5a1, the free-living diazotroph except some different base sequences recognized by BglII, PstI and SmaI. However, there were no differences detected among the 3 associative Klebsiella strains.

To determine whether nif structural gene fragments : nifH, nifD or nifK that give differences in hybridization pattern, Fa (3.32 kb : nifH and part of nifD), Fb (1.45 kb : part of nifDK) and Fc (5.24 kb : nifKTY and part of nifE) (Figure 1.1 c) were prepared by recovering from low-melting temperature agarose gel electrophoresis after cutting pSA30 with BamHI and HindIII as shown in Figure 3.12. The per cent recovery

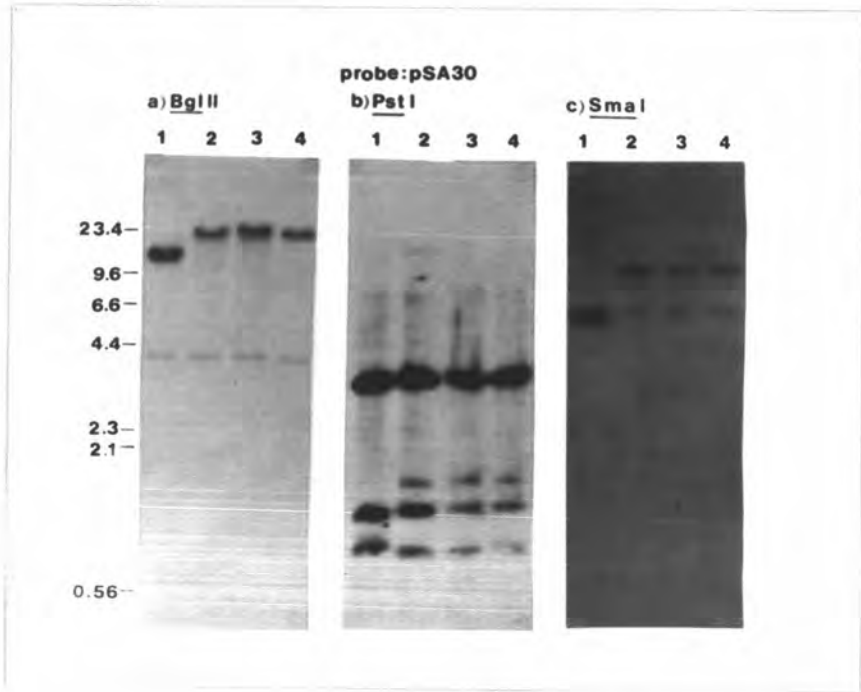


Figure 3.11. Southern hybridization between pSA30 probe and chromosomal DNA of Klebsiella spp. digested with BglII, PstI and SmaI.

The chromosomal DNA (1.5 µg) of Klebsiella spp. were digested with (a) BglII, (b) PstI and (c) SmaI, and hybridized with ^{32}P-pSA30 probe (specific activity of 8.34 x 10^8 cpm/µg DNA and 4.17 x 10^8 cpm was added). The hybridization was performed at 37°c for 24 h. The filter was washed at 55°c and exposed to X-ray film at -70°c for 2-4 h with intensifying screens.

lane 1 : K. pnemoniae M5a1

lane 2 : K. oxytoca NG13

lane 3 : K. R15

lane 4 : K. R17

Table 3.1 Restriction analysis of *nif* structural genes of *Klebsiella* strains by Southern hybridization.

Enzyme	Strain	pSA30 probe (*nif* HDKTY)
BamHI	NG13, R15, R17	17.5, 4.3
	M5a1	17.5, 4.3
BglII	NG13, R15, R17	23.4, 3.9
	M5a1	14.0, 3.9
EcoRI	NG13, R15, R17	6.2
	M5a1	6.2
HindIII	NG13, R15, R17	21.0, 16.5
	M5a1	21.0, 16.5
PstI	NG13, R15, R17	3.4, 1.5, 1.2, 0.77
	M5a1	3.4, 1.2, 0.83
SalI	NG13, R15, R17	3.5, 2.4, 1.4
	M5a1	3.5, 2.4, 1.4
SmaI	NG13, R15, R17	9.6, 5.8
	M5a1	5.8, 5.5
XhoI	NG13, R15, R17	19.0
	M5a1	19.0

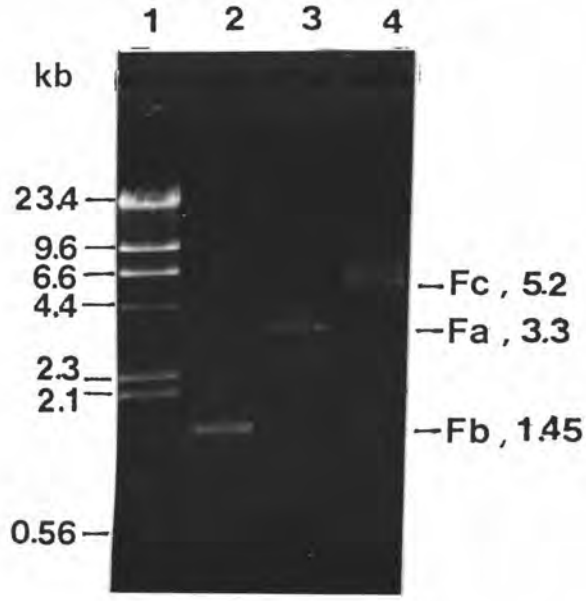


Figure 3.12. Electrophoresis of nif structural gene fragments obtained by cutting pSA30 with BamHI and HindIII and recovering from low-melting temperature agarose gel electrophoresis.

The nif structural gene fragments (Fa, Fb and Fc) were prepared from cutting pSA30 with BamHI and HindIII and then electrophoresed on low-melting temperature agarose gel. The recovery of DNA fragments were performed by extraction from melted gel with phenol.

lane 1 : λ/HindIII

lane 2 : Fb (1.45 kb)

lane 3 : Fa (3.32 kb)

lane 4 : Fc (5.24 kb)

of Fa, Fb and Fc fragments were 25, 90 and 23% respectively. These fragments were labelled with [α-^{32}P] dCTP by nick translation for maximum incorporation of 52% at 120 min (Fa), 27% at 60 min (Fb) and 96% at 30 min (Fc) as shown in Figure 3.13. Their specific activities were 6.78 x 10^7, 3.7 x 10^7 and 9.17 x 10^7 cpm/µg DNA respectively. The restriction patterns of Fa, Fb and Fc fragments hybridized with chromosomal DNA on BglII, PstI or SmaI cutting are shown in Figure 3.14 and fragments sizes are summarized in Table 3.2. Analysis from these differences in fragment sizes and known restriction map of BglII, PstI and SmaI in the nif genes region of K. pneumoniae as shown in Figure 3.15, the alteration in base sequences observed in associative strains could reside in nifL, nifE and nifJ regions as derived from larger fragment hybridized to all Fa, Fb and Fc in BglII cutting, variable length hybridized to Fc in PstI cutting, and larger fragment hybridized to all Fa, Fb and Fc in SmaI cutting.

3.5. Comparison of RFLP by glnA probe.

To determine the extent of homology between free-living K. pneumoniae M5a1 and associative Klebsiella strains in glnA region, pAM51 plasmid (Figure 1.2b) was purified and checked for correct restriction patterns as shown in Figure 3.16. The maximum incorporation of [α-^{32}P] dCTP into pAM51 was 75% at 30 min as shown in Figure 3.17 and its specific activity was 3.08 x 10^8 cpm/µg DNA. Using ^{32}P-pAM51 as probe, there were non-specific binding not only with chromosomal DNA but also with λ/HindIII fragments (data not shown), therefore only glnA region (0.86 kb) of pAM51 was recovered from low-melting temperature agarose gel electrophoresis at per cent recovery of 98 after cutting pAM51 with EcoRI as shown in Figure 3.18 and then labelled with [α-^{32}P] dCTP for maximum incorporation at 28% of 60 min

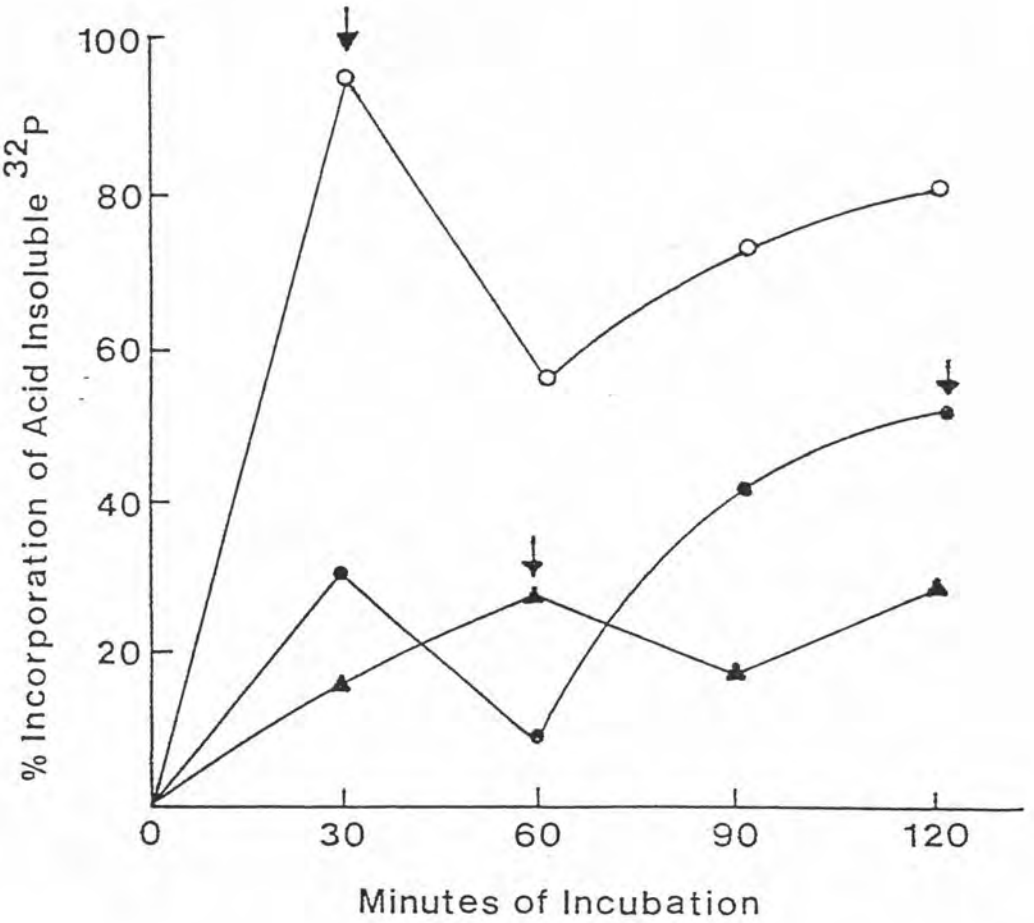


Figure 3.13. Time course of nick translation reaction into Fa, Fb and Fc fragments of pSA30.

The 500 ng of DNA fragments : Fa (●), Fb (▲) and Fc (○), were labelled with 100 µCi [α-^{32}P] dCTP in the 30 µl reaction mixture containing 13.3 µM of dNTPs (dATP, dGTP and dTTP each), 1.2 units of DNA polymerase I and 20 microunits of Dnase I. The nick translation reaction was performed at 13°c for 120 min. The maximum per cent incorporation of [α-^{32}p] dCTP into DNA resulted in the specific activity of final product : Fa, 6.78 x 10^7; Fb, 3.7 x 10^7 and Fc, 9.17 x 10^7 cpm/µg DNA.

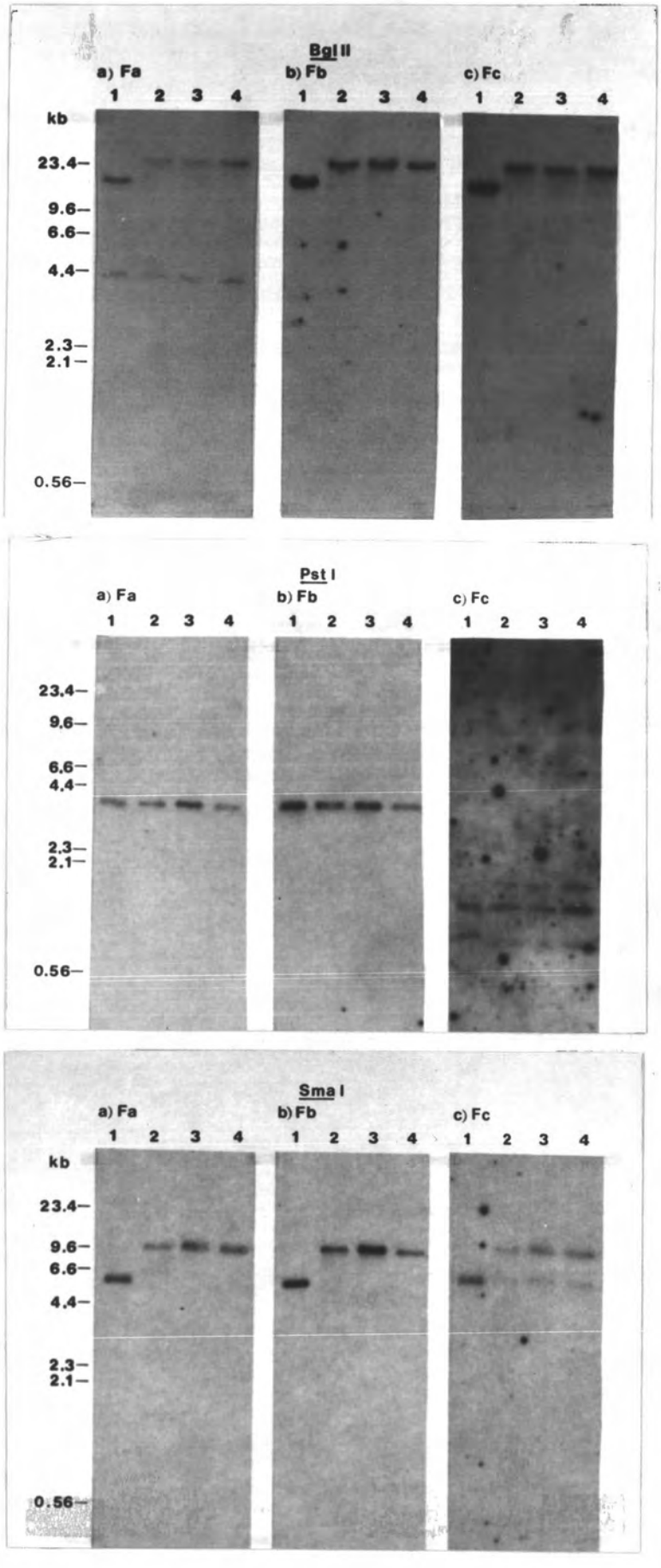


Figure 3.14. Southern hybridization of nif structural genes fragments (Fa, Fb and Fc) with chromosomal DNA of Klebsiella spp. digested by BglII, PstI and SmaI.

The chromosomal DNA (1.5 µg) of Klebsiella spp. were digested with BglII, PstI, SmaI and hybridized with the probes indicated: column a) Fa, column b) Fb and column c) Fc (specific activity 6.78, 3.70 and 9.17 x 10^7 cpm/µg DNA and 2.26, 1.23, and 3.06 x 10^7 cpm were added respectively). The hybridization was performed at 37°c for 24 h. The filter was washed at 55°c and exposed to X-ray film at -70°c for 1-2 d with intensifying screens.

lane 1 : K. pnemoniae M5a1

lane 2 : K. oxytoca NG13

lane 3 : K. R15

lane 4 : K. R17

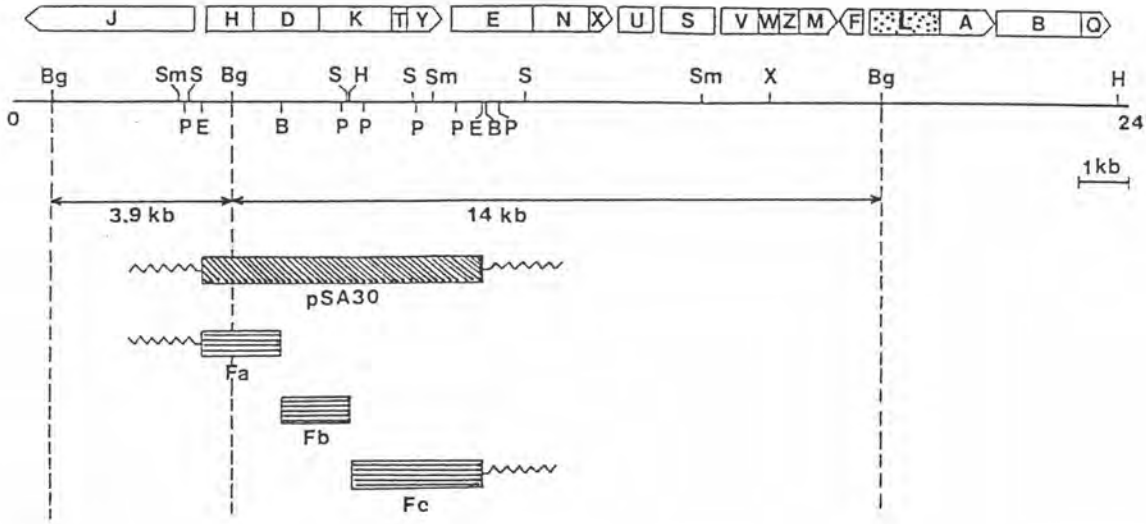
Table 3.2 Restriction analysis of *nif* structural genes of *Klebsiella* strains by Southern hybridization using *nif* structural gene fragments as probes.

Enzyme	Strain	Fa probe (*nif* HD)	Fb probe (*nif* DK)	Fc probe (*nif* KTY)
*Bgl*II	NG13, R15, R17	23.4, 3.9	23.4	23.4
	M5a1	14.0, 3.9	14.0	14.0
*Pst*I	NG13, R15, R17	3.4	3.4	1.5,1.2, 0.77
	M5a1	3.4	3.4	1.2,0.83
*Sma*I	NG13, R15, R17	9.6	9.6	9.6,5.8
	M5a1	5.5	5.5	5.8,5.5

Figure 3.15. Hybridization scheme between *nif* genes of *K. pneumoniae* and pSA30, Fa, Fb and Fc probes.

The restriction map of *nif* genes of *K. pneumoniae* cut with (a) *Bgl*II, (b) *Pst*I and (c) *Sma*I were shown altogether with probes : pSA30, Fa, Fb and Fc.

a)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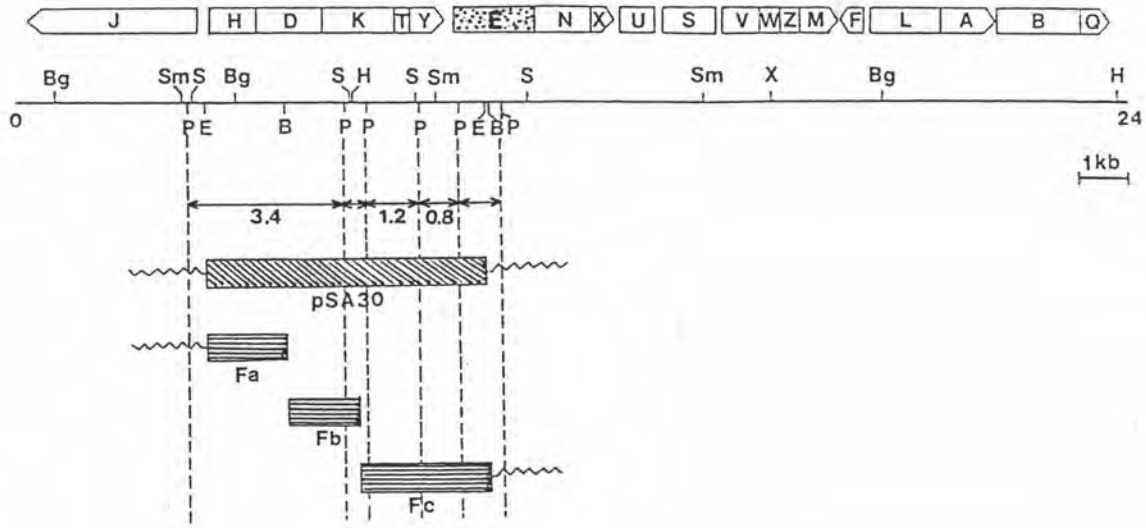
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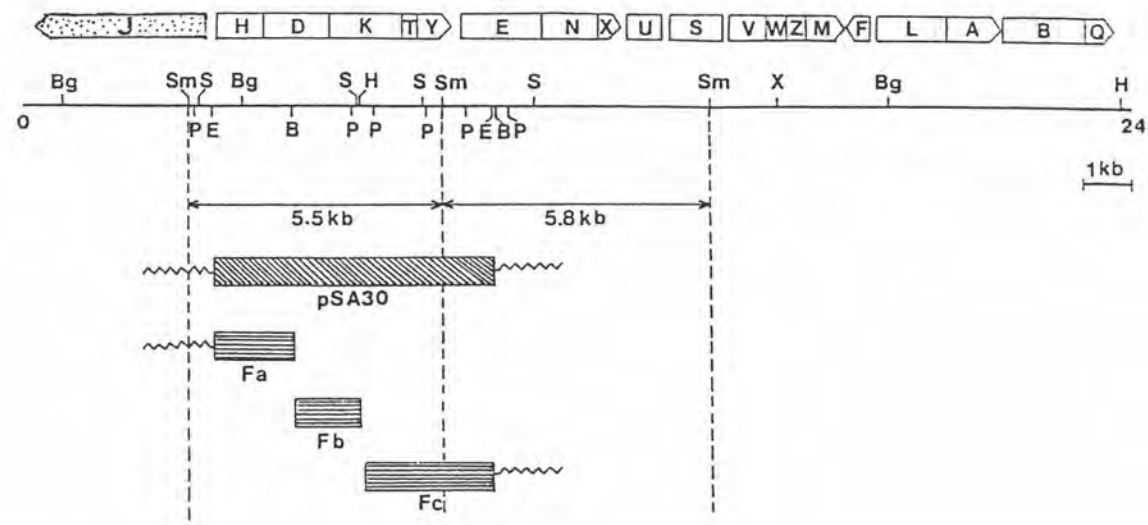
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pSA30
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Fb
Fc

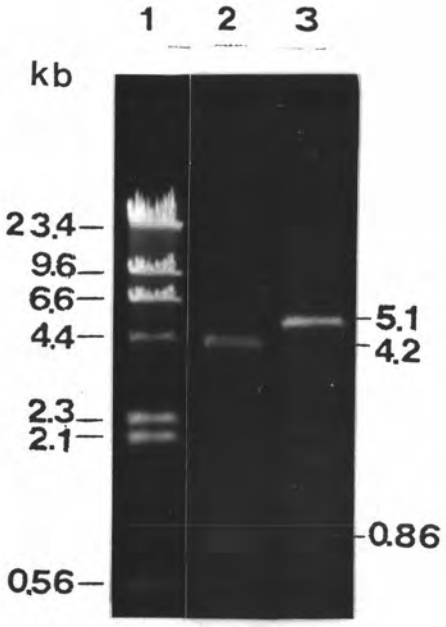


Figure 3.16. Restriction patterns of plasmid pAM51 on agarose gel.

The plasmid pAM51 digested with EcoRI or SmaI were loaded on 0.7% agarose gel and electrophoresed in tris-borate buffer, pH 8.3 at 80 volt for 2 h.

lane 1 : λ/HindIII

lane 2 : pAM51/EcoRI

lane 3 : pAM51/SmaI

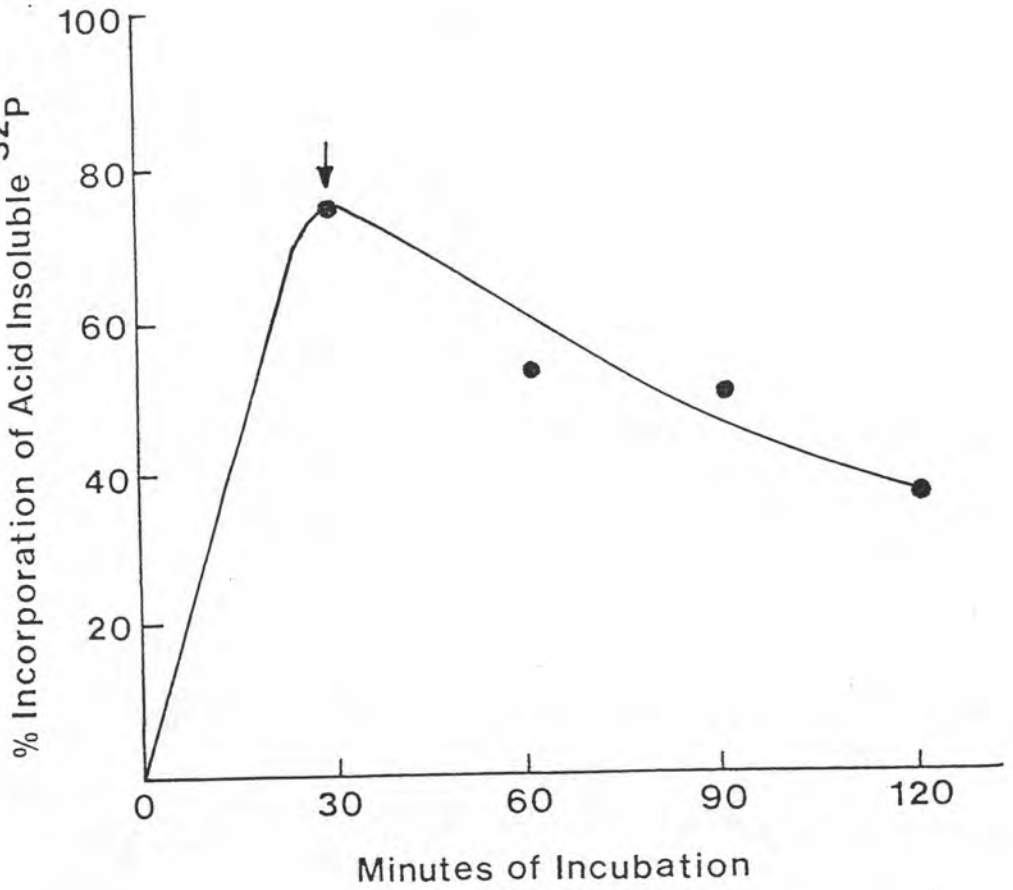


Figure 3.17. Time course of nick translation reaction into pAM51.

The plasmid pAM51 (500 ng) was labelled with 100 µCi [α-^{32}P] dCTP in 30 µl reaction mixture containing 13.3 µM of dNTPs (dATP, dGTP and dTTP each), 1.2 units of DNA polymerase I and 20 microunits of Dnase I. The nick translation reaction was performed at 13°c for 120 min. The maximum per cent incorporation of [α-^{32}p] dCTP into pAM51 resulted in the specific activity of 3.08×10^8 cpm/µg DNA.

Figure 3.18. Patterns of glnA fragment after cutting pAM51 with EcoRI and recovering from low-melting temperature agarose gel electrophoresis.

The glnA fragment was prepared from cutting pAM51 with EcoRI and then electrophoresed on low-melting temperature agarose gel. The recovery of glnA fragment was performed by extraction from melted gel with phenol.

lane 1 : λ/HindIII

lane 2 : pACYC 184 (4.2 kb)

lane 3 : glnA fragment (0.86 kb)

(Figure 3.19) and specific activity of 2.26 x 10^7 cpm/µg DNA. Figure 3.20 shows that only XhoI cutting results in different restriction fragment sizes between free-living K. pneumoniae M5a1 and the associative Klebsiella strains : K. oxytoca NG13, K. R15 and K. R17 as summarized in Table 3.3. All other 5 enzymes (EcoRI, PstI, SalI, SmaI and HindIII) with restriction sites within and near glnA ntrBC region indicate very conserved sequences in this region among all strains of Klebsiella tested, because extra hybridizing fragment has not been observed. Since, XhoI restriction site is not in 11.1 kb fragment containing glnA ntrB ntrC operon, the difference in restriction pattern detected by XhoI cutting is not in this important NTR regulatory system, but could be upstream or downstream from this region within 6 kb.

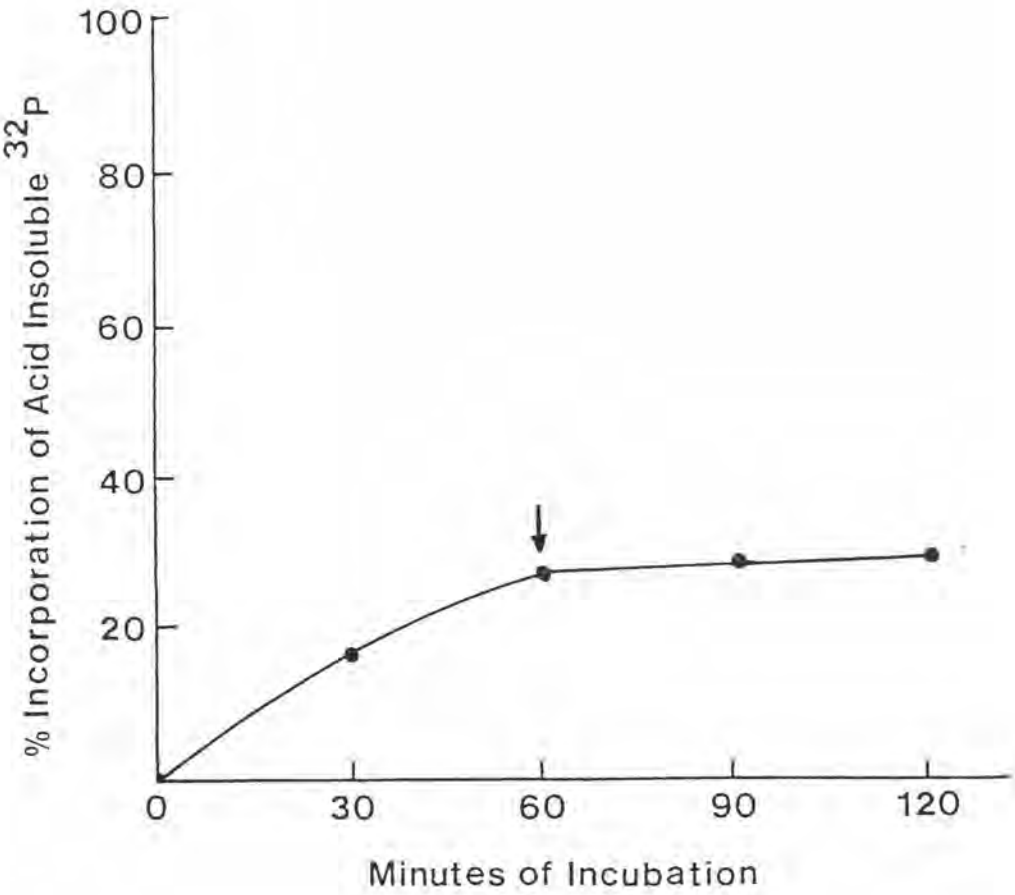
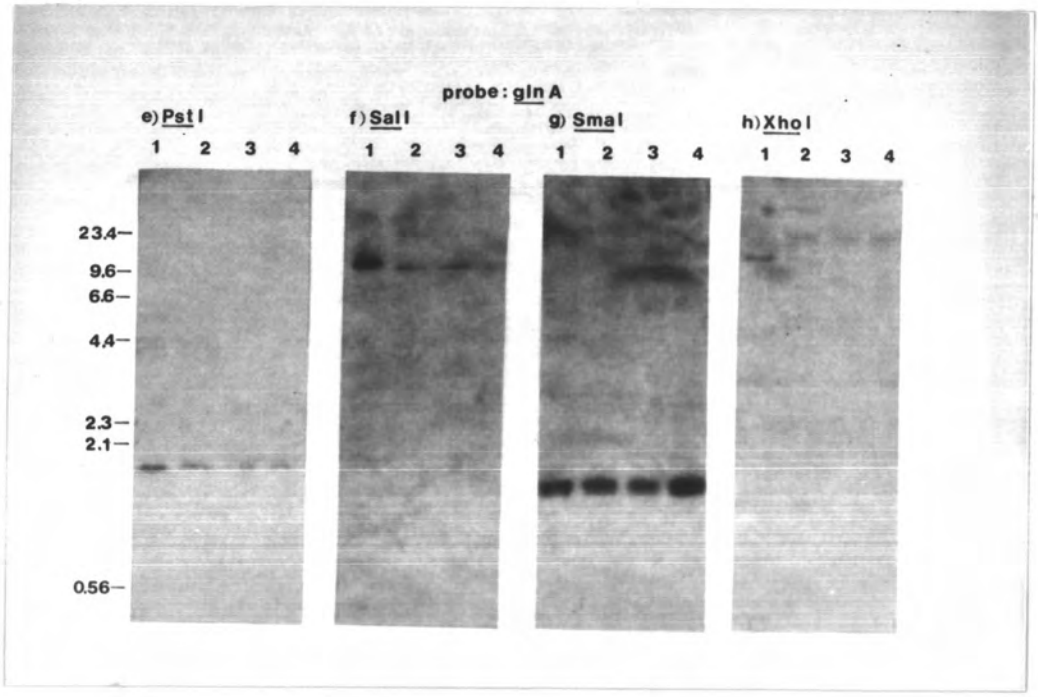


Figure 3.19. Time course of nick translation reaction into glnA probe. The glnA gene (500 ng) was labelled with [α-^{32}P] dCTP in 30 µl reaction mixture containing 13.3 µM of dNTPs (dATP, dGTP and dTTP each), 1.2 units of DNA polymerase I and 20 microunits of Dnase I. The nick translation reaction was performed at 13°c for 120 min. The maximum per cent incorporation of [α-^{32}p] dCTP into glnA resulted in the specific activity of 2.26 x 10^7 cpm/µg DNA.


probe: glnA
e) PstI
f) SalI
g) SmaI
h) XhoI
1 2 3 4
23.4
9.6
6.6
4.4
2.3
2.1
0.56

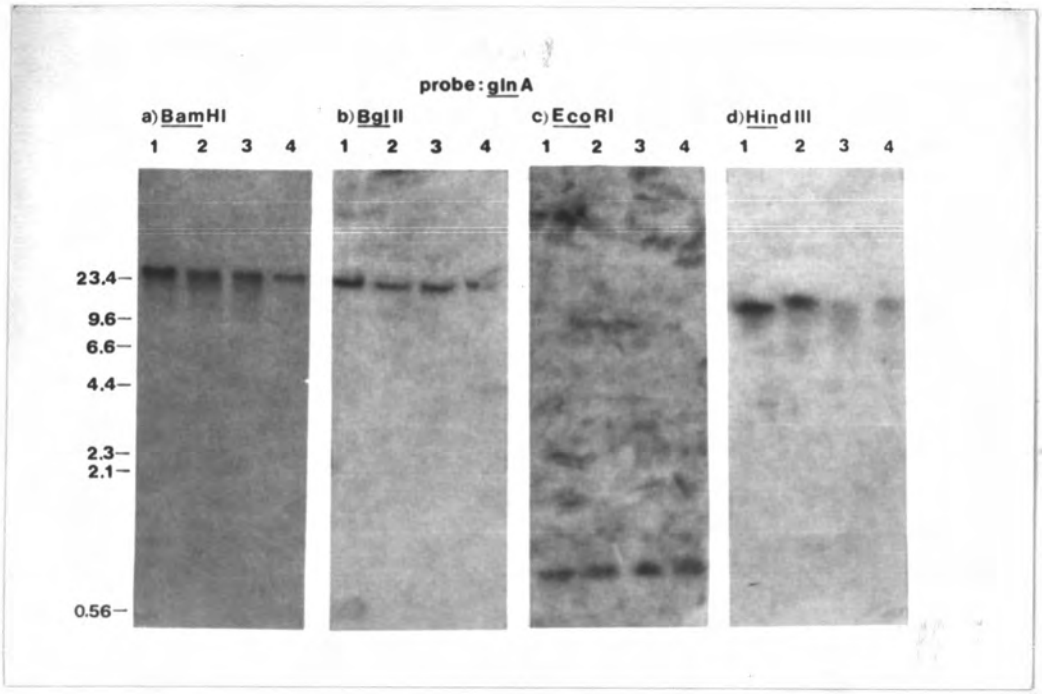

probe: glnA
a) BamHI
b) BglII
c) EcoRI
d) HindIII
1 2 3 4
23.4
9.6
6.6
4.4
2.3
2.1
0.56

Table 3.3 Restriction analysis of gln A gene of Klebsiella strains by Southern hybridization

Enzyme	Strain	gln A probe
BamHI	NG13, R15, R17	17.5
	M5a1	17.5
BglII	NG13, R15, R17	21.0
	M5a1	21.0
EcoRI	NG13, R15, R17	0.86
	M5a1	0.86
HindIII	NG13, R15, R17	11.2
	M5a1	11.2
PstI	NG13, R15, R17	1.9
	M5a1	1.9
SalI	NG13, R15, R17	11.0
	M5a1	11.0
SmaI	NG13, R15, R17	1.55, 1.45
	M5a1	1.55, 1.45
XhoI	NG13, R15, R17	23.4
	M5a1	17.0