CHAPTER III

RESULTS

3.1. Analysis of DNA purity.

Purified plasmid DNA (pE39, pRmSL42 and pSA30) were amplified by transforming into *Escherichia coli* strain HB101 cells and was isolated by rapid alkaline extraction. The plasmid DNA was further purified from contaminated RNA and high molecular weight DNA by isopycnic centrifugation in cesium chloride gradient containing ethidium bromide. The purity of plasmids (pE39, pRmSL42 and pSA30) were evident by $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8-2.0, and no visible RNA contamination when electrophoresed. Figure 3.1. confirmed the purity of each plasmid prepared for using as the DNA probe by restriction patterns.

Isolation of chromosomal DNAs from *A. brasilense* Sp7, *B. japonicum* THA5, THA7, *K. oxytoca* R15, R17, NG13, *K. pneumoniae* M5a1, *R. leguminosarum* TAL1402, *R. meliloti* TAL380, TAL 1372 according to Rodriquez and Tsit (1983), yielded high molecular weight DNA without RNA bands as shown in Figure 3.2. and the $A_{260\ nm}/A_{280\ nm}$ ratio were always higher than 1.7.

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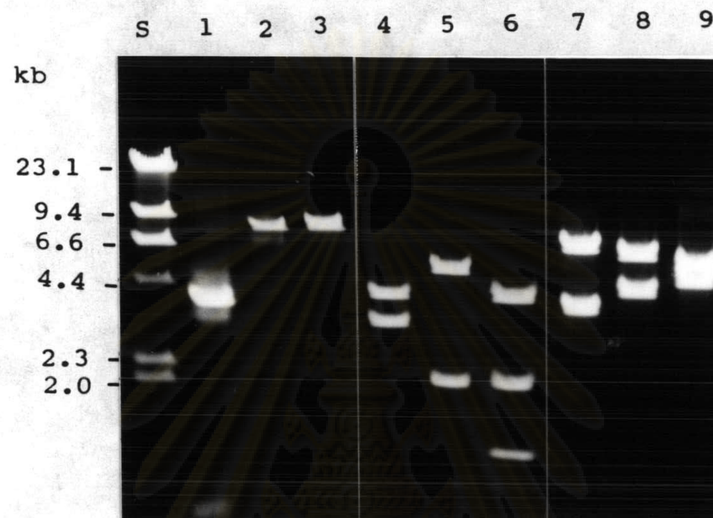

Figure 3.1. Electrophorogram of purified plasmid pE39, pRmSL42 and pSA30 after digestion with restriction enzymes.

lane S : λ/*Hind*III

lane 1 : pE39/*Bam*HI (0.6, 2.7 kb)

lane 2 : pRmSL42/*Bam*HI (8.5 kb)

lane 3 : pRmSL42/*Eco*RI (8.5 kb)

lane 4 : pRmSL42/*Bam*HI and *Eco*RI (5.2, 3.3 kb)

lane 5 : pRmSL42/*Bam*HI and *Hind*III (6.3, 2.2 kb)

lane 6 : pRmSL42/*Bam*HI, *Eco*RI and *Hind*III (1.3, 2.2, 5.0 kb)

lane 7 : pSA30/*Bam*HI (6.5, 3.7 kb)

lane 8 : pSA30/*Eco*RI (6.1, 4.2 kb)

lane 9 : pSA30/*Hind*III (5.4, 4.7 kb)

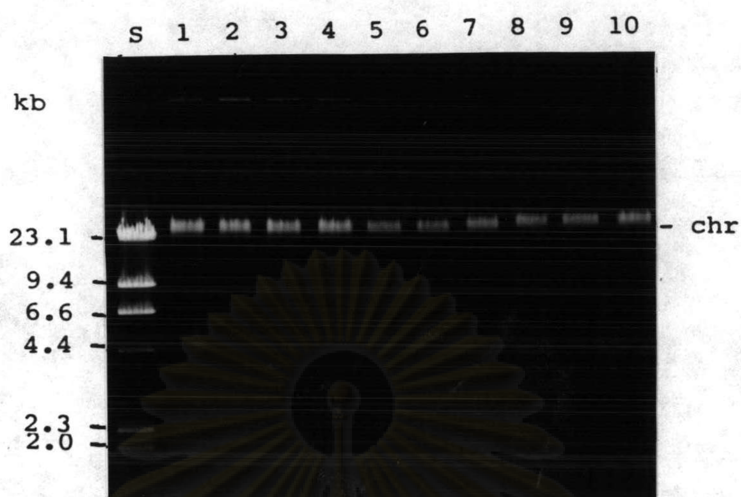

Figure 3.2. High molecular weight chromosomal DNA from various strains of *Klebsiella*, *Azospirillum*, *Rhizobium* and *Bradyrhizobium*.

Three 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 hr.

lane S : λ/*Hind*III

lane 1 : *K. oxytoca* R15

lane 2 : *K. oxytoca* R17

lane 3 : *K. oxytoca* NG13

lane 4 : *K. pneumoniae* M5a1

lane 5 : *A. brasilense* Sp7

lane 6 : *R. meliloti* TAL380

lane 7 : *R. meliloti* TAL1372

lane 8 : *R. leguminosarum* TAL1402

lane 9 : *B. japonicum* THA5

lane 10: *B. japonicum* THA7

3.2. Restriction endonuclease digestion of chromosomal DNA.

In this study, selected type II restriction endonucleases were; *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Xho*I because their recognition sequence of six nucleotides are known to be present in the plasmid DNA used as probes

Chromosomal DNA 1.0 μg from each bacterial strain was digested with 10 units or 20 units of each restriction enzyme at 37 °C (except *Sma*I at 30 °C) overnight. Figure 3.3. shows that most of isolated DNA were completely digested by either 10 or 20 units of every enzyme tested, although a few show rather large fragments when digested with 10 units, therefore 10 units/μg DNA, ratio of restriction enzyme per μg DNA was sufficient, and 4 μg of chromosomal DNA was used for digestion and 3 μg DNA was electrophoresed on a 100 x 90 x 6 mm agarose gel and photographed after ethidium bromide staining. Figure 3.4. shows electrophorogram among digested DNA from various genera of N_2-fixing bacteria.

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10 units | 20 units

S 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10

kb

23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

A) *Bam*HI

B) *Bgl*II

C) *Eco*RI

D) *Hind*III

10 units 20 units

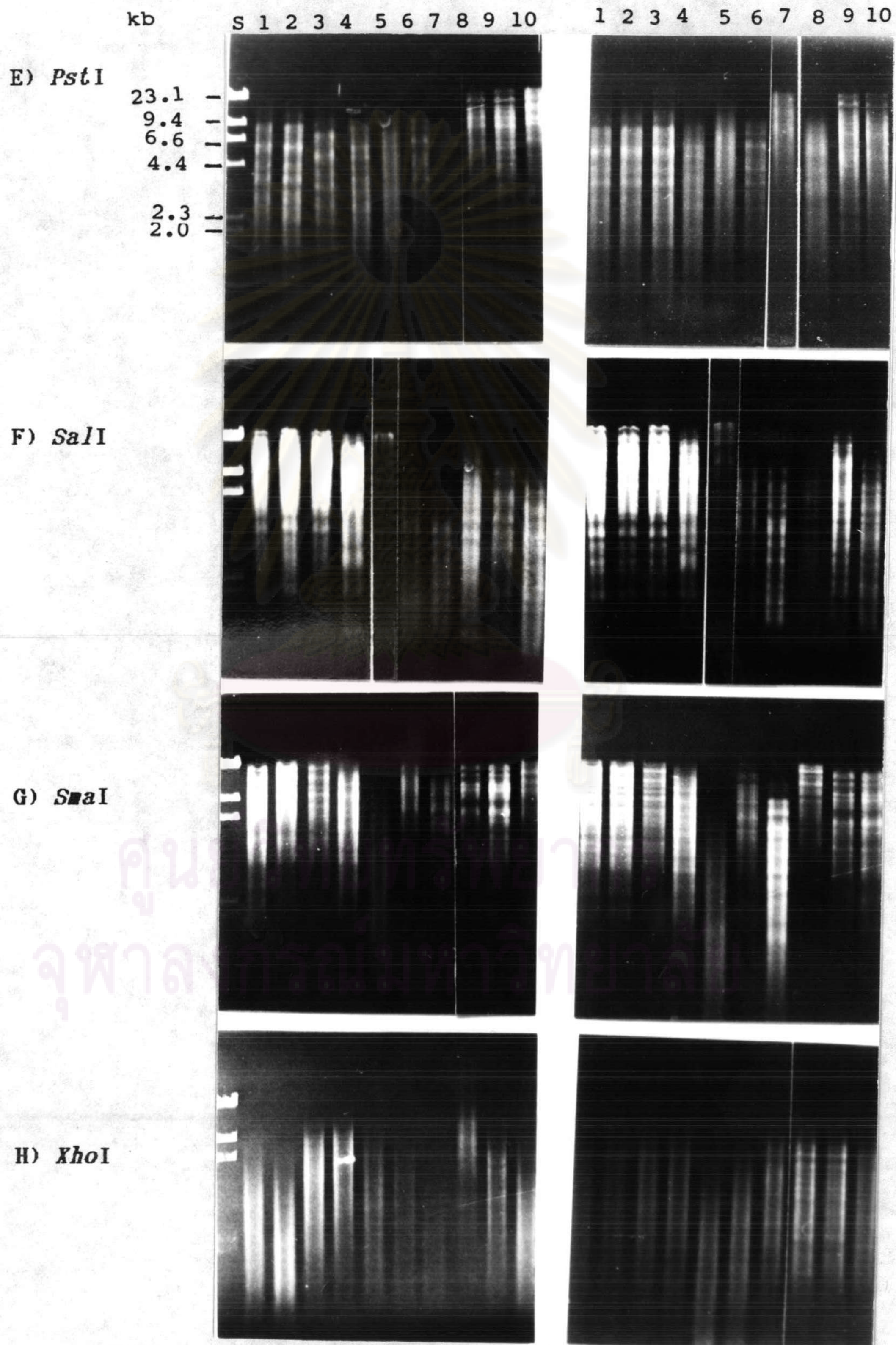

Figure 3.4. Electrophorogram of 3 μg chromosomal DNA of 10 strains of N_2-fixing bacteria digested with 8 restriction enzymes (40 units/4 μg DNA) on 0.7% agarose gel (100 x 90 x 6 mm) and electrophoresed in Tris-borate buffer, pH 8.3 at 80 volts for 3 hr.

lane S : λ/*Hind*III

lane 1 : *K. oxytoca* R15

lane 2 : *K. oxytoca* R17

lane 3 : *K. oxytoca* NG13

lane 4 : *K. pneumoniae* M5a1

lane 5 : *A. brasilense* Sp7

lane 6 : *R. meliloti* TAL380

lane 7 : *R. meliloti* TAL1372

lane 8 : *R. leguminosarum* TAL1402

lane 9 : *B. japonicum* THA5

lane 10: *B. japonicum* THA7

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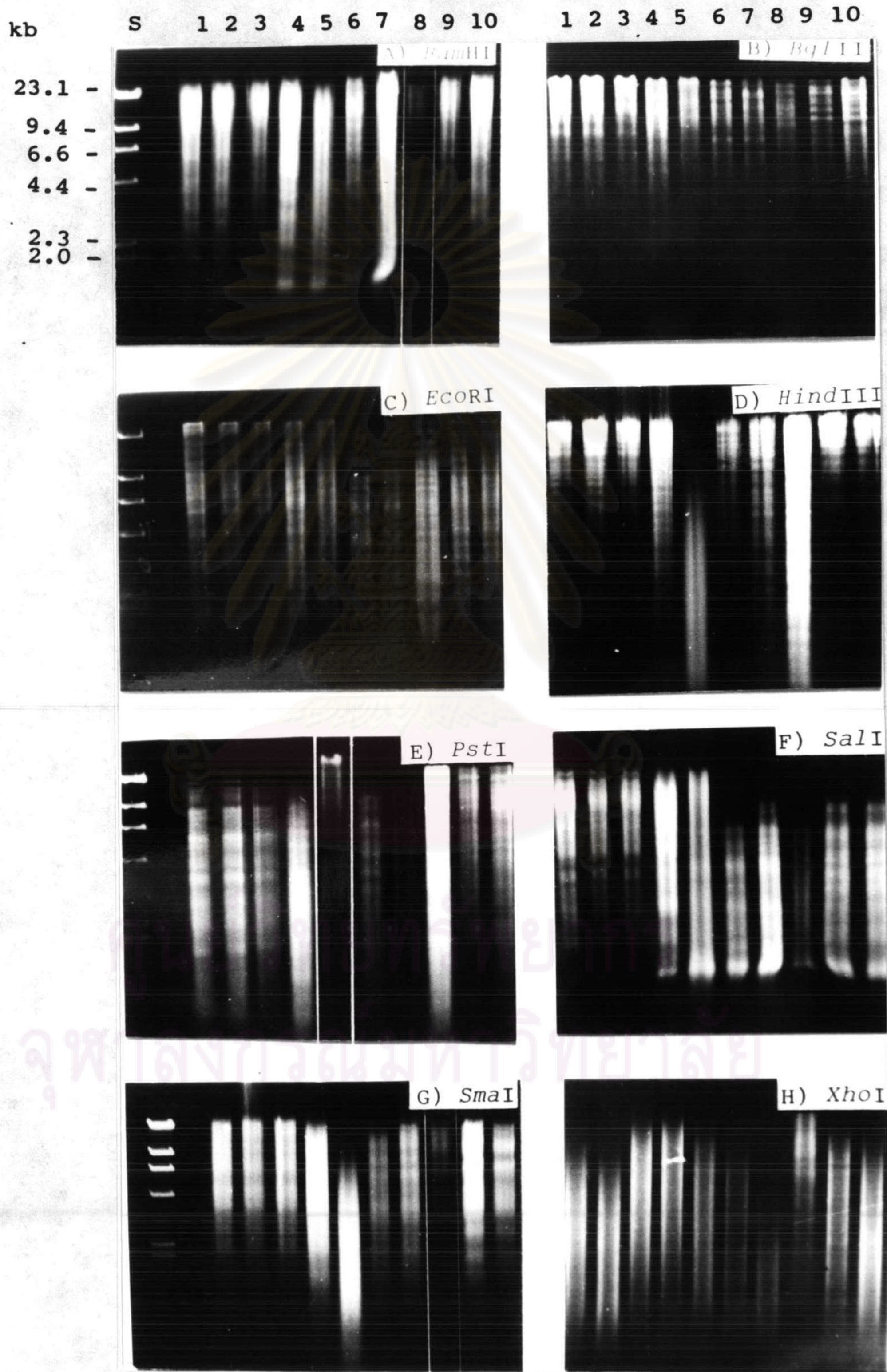
kb
S
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -
A) BamHI
B) BglII
C) EcoRI
D) HindIII
E) PstI
F) SalI
G) SmaI
H) XhoI

3.3. Preparation of purified DIG-labeled DNA probes by random primer.

To find out whether there is a DNA sequence homology between common *nod* genes of *R. meliloti*, *nif* structural genes of *K. pneumoniae* and chromosomal DNA of these N_2-fixing bacteria, the purified pE39 containing *nod*D1 of *R. meliloti* 1021 digested with *Bam*HI, the pRmSL42 containing *nod*ABC of *R. meliloti* 1021 digested with *Bam*HI, *Eco*RI and *Hind*III and the pSA30 containing *nif*HDKYT of *K. pneumoniae* M5a1 digested with *Eco*RI were separated from their vectors by recovering the inserted genes from low-melting temperature gel electrophoresis (Figure 3.5.); pE39 contained 0.6 kb:*nod*D1, 2.7 kb:pUC8, pRmSL42 contained 1.3 kb:*nod*C, 2.2 kb:*nodAB*, 5.0 kb:pBR322 and pSA30 contained 4.2 kb:pACYC84, 6.1 kb:*nif*HDK, and labeled genes (0.6 kb: *nod*D1, 1.3 kb:*nod*C, 2.2 kb:*nod*AB, and 6.1 kb:*nif*HDK) with DIG-dUTP by random primer reaction.

3.4. Dot hybridization by *nod*D1, *nod*AB, *nod*C and *nif*HDK probes

Figure 3.6. shows detection of the specific activity of labeled DNA probe by dot hybridization where *nif*HDK probe shows the highest specific activity at 1 pg/μl, followed by *nod*AB at 10 pg/μl and *nod*C only at 100 pg/μl when compared with control labeled DNA (pBR328) provided in the labeling kit. As for *nod*D1 the apparently high specific activity of 10 pg/μl resulted from initial amount of the labeled DNA was twice of the other probes.

Dot hybridization of each labeled probe: *nod*D1, *nod*AB, *nod*C and *nif*HDK with the chromosomal DNA of N_2-fixing bacteria were shown

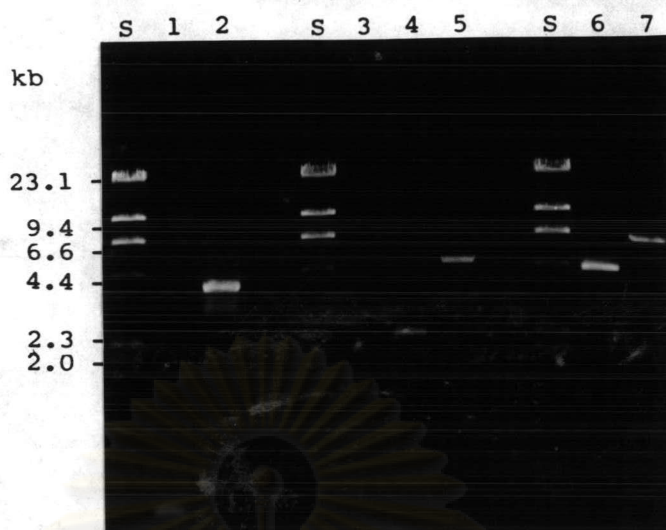

Figure 3.5. Electrophorogram of *nod* gene and *nif* gene fragments obtained by cutting pE39 with *Bam*HI, cutting pRmSL42 with *Bam*HI, *Eco*RI and *Hind*III and cutting pSA30 with *Eco*RI and recovering from low-melting temperature agarose gel electrophoresis.

The *nod*D1 gene fragments (0.6 and 2.7 kb) were prepared by cutting pE39 with *Bam*HI, the *nod*ABC gene fragments (1.3, 2.2 and 5.0 kb) were prepared by cutting pRmSL42 with *Bam*HI, *Eco*RI and *Hind*III and the *nif*HDK gene fragments (4.2 and 6.1 kb) were prepared by cutting pSA30 with *Eco*RI and then electrophoresed on low-melting temperature agarose gel. The recovery of DNA fragments were performed by extracting the melted gel with phenol.

lane S : λ/*Hind*III

lane 1 : 0.6 kb:*nod*D1 ┐ pE 39
lane 2 : 2.7 kb:pUC8 ┘

lane 3 : 1.3 kb: *nod*C ┐
lane 4 : 2.2 kb:*nod*AB ├ pRmSL42
lane 5 : 5.0 kb:pBR322 ┘

lane 6 : 4.2 kb:pACYC184 ┐ pSA30
lane 7 : 6.1 kb:*nif* HDK ┘

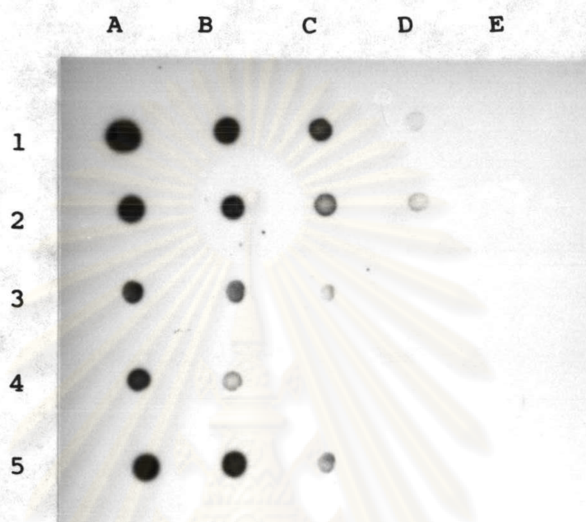

Figure 3.6. Dot hybridization experiment to evaluate the specific activity of labeled probe

(1) control labeled DNA:pBR328, (2) *nif*HDK:6.1 kb labeled DNA, (3) *nod*AB:2.2 kb labeled DNA, (4) *nod*C:1.3 kb labeled DNA and (5) *nod*D1:0.6 kb, the specific activity of labeled DNA; (A) 1ng/μl, (B) 100 pg/μl, (C) 10 pg/μl, (D) 1 pg/μl and (E) 0.1 pg/μl.

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in Figure 3.7. indicating that the amount of DNA used was critical, since 2 μg and 3 μg DNA, resulted in different patterns. Only 3 μg DNA, that DNA sequence homology with the *nod*D1 gene can be detected in associative *Klebsiella*, *Azospirillum*, although *nod*D1 homology can be detected in all the *Rhizobium* spp. at 2 μg DNA. *K. pneumoniae* M5a1, the free-living N_2-fixing bacteria failed to show *nod*D1 homology at either DNA concentration. At 2 μg DNA, only *R. meliloti* TAL380 and TAL1372 show hybridizable bands with *nod*AB and *nod*C, but when the amount of DNA was increased to 3 μg, the associative *Klebsiella*, *K. pneumoniae* M5a1, *A. brasilense* and other *Rhizobium* spp. showed very faint hybridization spots with the *nod*AB and *nod*C probes, where *nod*C homology in 3 μg of chromosomal DNA of *R. leguminosarum* strain TAL1402 show higher density than *nod*AB probes. The chromosomal DNA of associative and free-living, *Klebsiella* strains have strong DNA sequence homology with the *nif*HDK probe at 2 μg DNA per spot, but other strains of N_2-fixing bacteria required 3 μg DNA for detection of *nif*HDK homology. However, dot hybridization is a procedure that enable rapid screening of high homology by passing electrophoresis and DNA fragment transfer, but give no information on genes organization.

3.4. Comparision of RFLPs by *nod* genes and *nif* genes

Southern hybridization of these labeled probes with their homologous nonlabeled genes were performed to test for its specificity (Figure 3.8.). The result indicated that each probe hybridized

Figure 3.8. Southern hybridization of *nod*D1, *nod*AB, *nod*C and *nif*HDK probes with nonlabeled genes, which were used as probe

The DNA (200-500 ng) of nonlabled genes; *nod*D1, *nod*AB, *nod*C and *nif*HDK and hybridized with DIG-*nod*D1, DIG-*nod*AB, DIG-*nod*C, DIG-*nif*HDK probes (amount of 10 ng/ml) at 65-68 $^\circ$C for 24 hr. The membrane was exposed to X-ray film at 37 $^\circ$C for 30 min intensifying screens.

lane S : λ/*Hind*III

lane 1 : *nod*D1 0.6 kb

lane 2 : *nod*C 1.3 kb

lane 3 : *nod*AB 2.2 kb

lane 4 : *nif*HDK 6.1 kb

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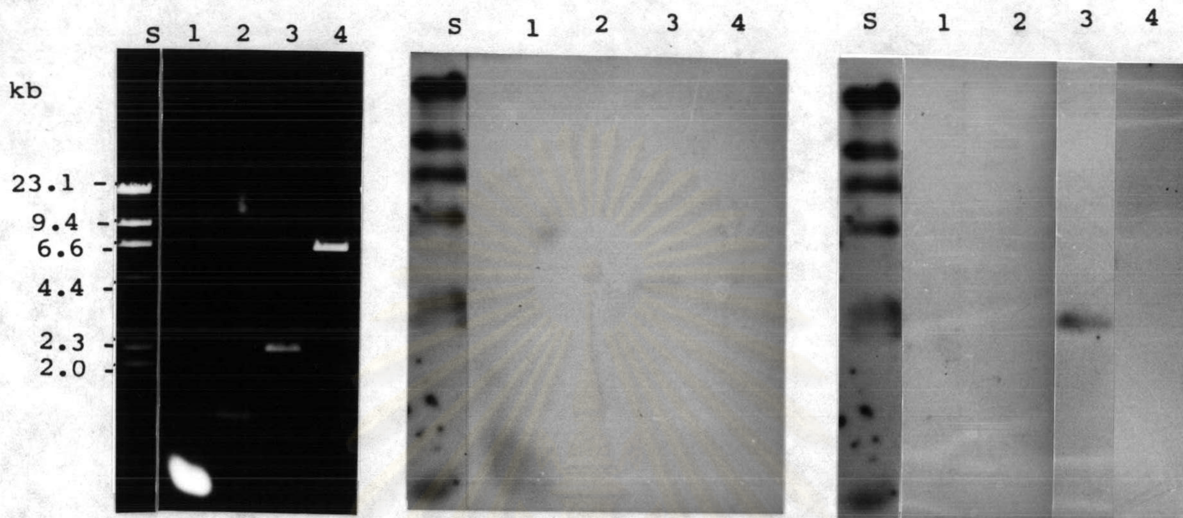
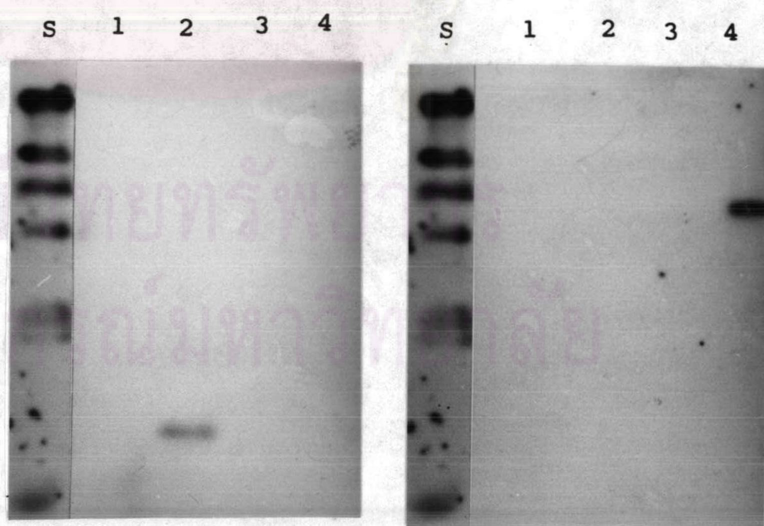

probe:*nod*D1
probe:*nod*AB
S 1 2 3 4
kb
23.1
9.4
6.6
4.4
2.3
2.0
S 1 2 3 4
S 1 2 3 4


probe:*nod*C
probe:*nif*HDK
S 1 2 3 4
S 1 2 3 4

specifically only with itself except *nod*D1, that cross hybridized with *nod*AB because of their contact origin. Using 3 μg of chromosomal DNA digested with restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Xho*I, the patterns and sizes of hybridization bands were compared according to the standard calibration graph (Appendix III) using *Hind*III digested lambda-DNA (2.0-23.1 kb) as standard molecular weight markers. The digested chromosomal DNA, were hybridized with *nod*D1 probe from *R. meliloti*. Figure 3.9. shows similar RFLP profiles between the two *R. meliloti* strains TAL380 and TAL1372 (lane 6 and 7) except *Bam*HI, *Pst*I and *Xho*I that can distinguish between these 2 strains. *Bgl*II can distinguish between *B. japonicum* strain THA5 and THA7. *B. japonicum*, *R.leguminosarum* and *R. meliloti* can be distinguished by *Bam*HI, *Hind*III and *Pst*I. The *Bam*HI digested DNA from associative *K. oxytoca* strain R15, R17 and NG13 show two DNA fragments of 4.0 and 4.9 kb that hybridize with *nod*D1 probe, which resemble other rhizobia strains tested and also similar to associative *A. brasilense* Sp7, but not the free-living *K. pneumoniae* M5a1 (Table 3.1.). Southern hybridization with *nod*AB probe and *nod*C probe failed to show any homology among 8 strains, and only showed similar RFLP patterns in two *R. meliloti* strains: TAL380 and TAL1372, confirming that these two genes organization are adjacent and strongly homologous in these 2 strains (Figure 3.10., 3.11. and Table 3.2., 3.3.). Although dot hybridization of 3 μg total chromosomal DNA of all N_2-fixing bacteria suggested for weak homology with the *nod*AB and *nod*C genes in various strains other

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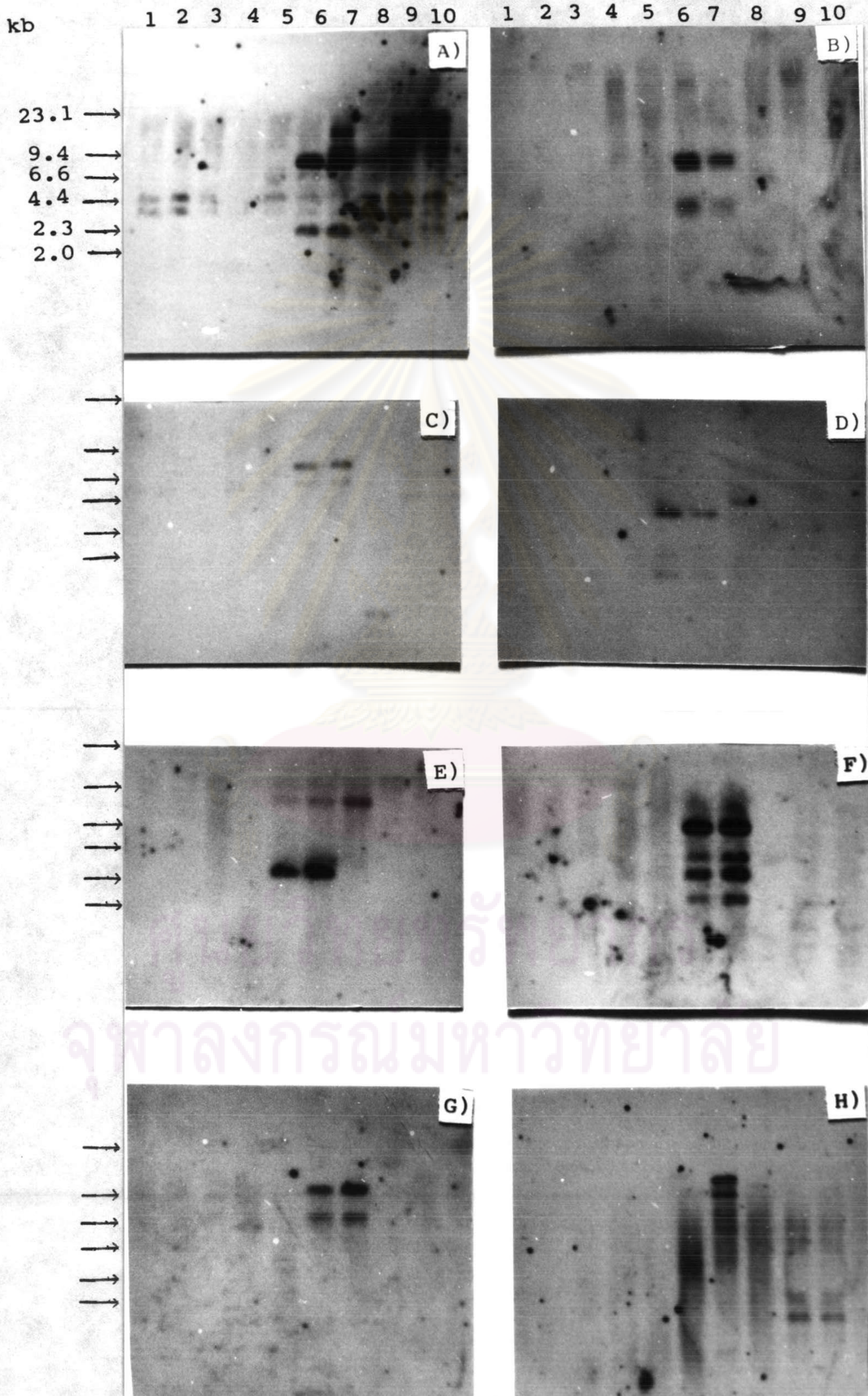
probe: nodD1
kb
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
23.1
9.4
6.6
4.4
2.3
2.0
A)
B)
C)
D)
E)
F)
G)
H)

Table 3.1. RFLP of *nod*D1 gene hybridized to 10 strains of N_2-fixing bacteria

Strain	Size of restriction fragment (kb)	
	A: *Bam*HI	B: *Bgl*II
R15, R17, NG13	4.9,4.0	-
M5a1	-	-
Sp7	6.8,4.9,4.0	-
TAL380	7.6,6.8,4.9,4.0,3.2	13.5,3.9,2.1
TAL1372	17.0,14.0,7.6,4.9,4.0,3.2	13.5,3.9,2.1
TAL1402	4.9,4.0,3.2	-
THA5	14.0,4.9,4.0,3.2	14.0,8.9
THA7	14.0,4.9,4.0,3.2	-
Strain	**C: *Eco*RI**	**D: *Hind*III**
R15, R17, NG13	-	-
M5a1	-	-
Sp7	-	-
TAL380	9.0,6.5	5.4,3.5,3.0
TAL1372	9.0,6.5	5.4,3.5,3.0
TAL1402	1.7	6.5
THA5	-	5.3
THA7	-	5.3
Strain	**E: *Pst*I**	**F: *Sal*I**
R15, R17, NG13	-	-
M5a1	-	-
Sp7	-	-
TAL380	16.0,10.8,4.1	6.0,4.5,3.7,3.0
TAL1372	16.0,10.8,4.8,4.1	6.0,4.5,3.7,3.0
TAL1402	16.0,10.8	-
THA5	22.0,14.5	2.3,1.9
THA7	22.0,14.5	2.3,1.9
Strain	**G: *Sma*I**	**H: *Xho*I**
R15, R17, NG13	-	-
M5a1	-	-
Sp7	-	-
TAL380	12.0,7.2	-
TAL1372	12.0,7.2	15.0,11.0
TAL1402	-	-
THA5	-	7.2,6.3,5.7,3.0,2.2
THA7	-	7.2,6.3,5.7,3.0,2.2

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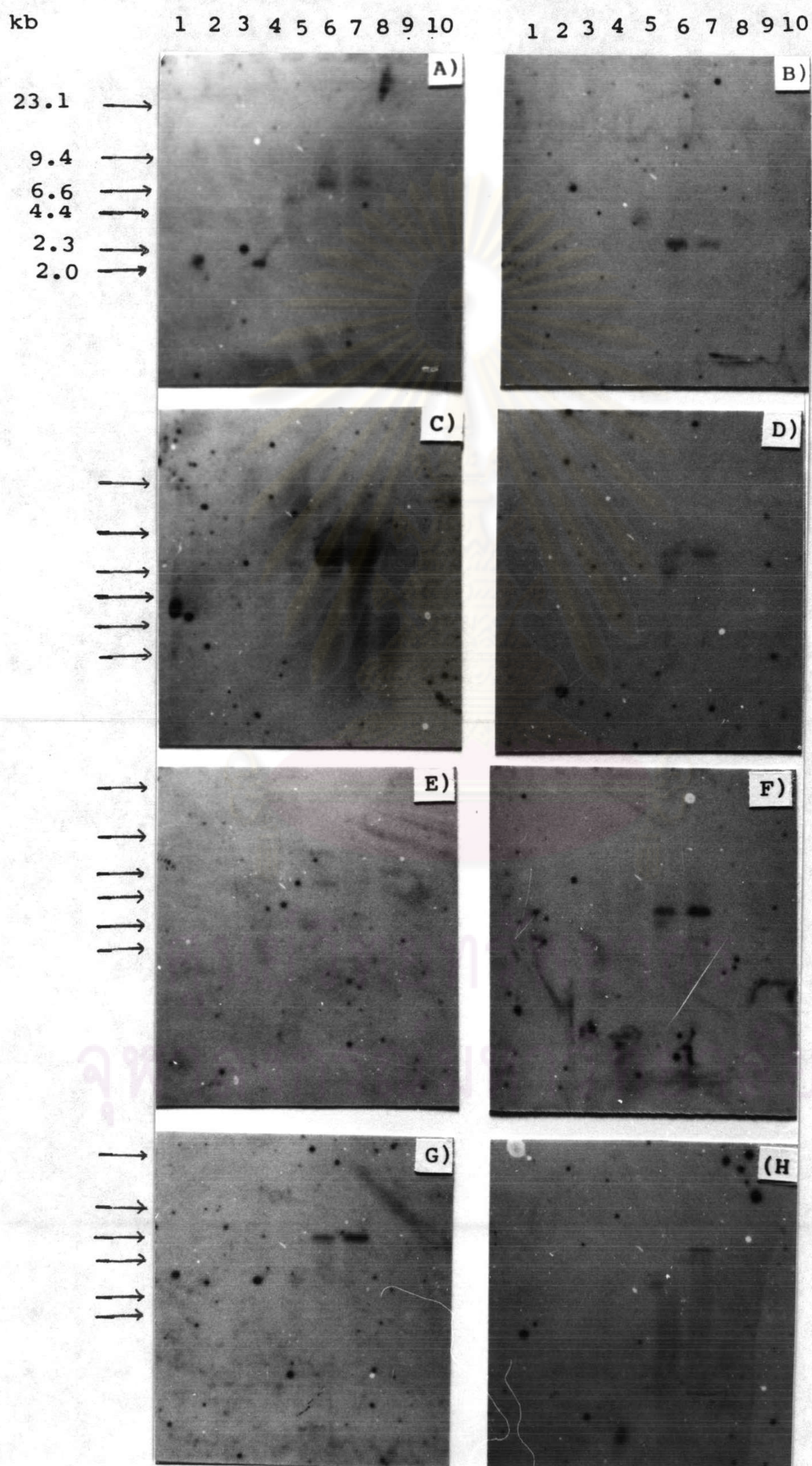
probe: nodAB
kb
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
23.1
9.4
6.6
4.4
2.3
2.0
A)
B)
C)
D)
E)
F)
G)
(H

Table 3.2. RFLP of *nod*AB gene hybridized to 10 strains of N_2-fixing bacteria

Strain	Size of restriction fragment (kb)							
	A: *Bam*HI	B: *Bgl*II	C: *Eco*RI	D: *Hind*III	E: *Pst*I	F: *Sal*I	G: *Sma*I	H: *Xho*I
R15, R17, NG13	-	-	-	-	-	-	-	-
M5a1	-	-	-	-	-	-	-	-
Sp7	-	-	-	-	-	-	-	-
TAL380	8.3	3.9	9.0,6.5	9.4	9.0,5.5	6.0,4.5,3.7	9.0	-
TAL1372	8.3	3.9	9.0,6.5	9.4	5.6	6.0,4.5,3.7	9.0	6.8
TAL1402	-	-	-	-	6.3	-	-	-
THA5	-	-	-	-	-	-	-	-
THA7	-	-	-	-	-	-	-	-

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probe: *nod*C

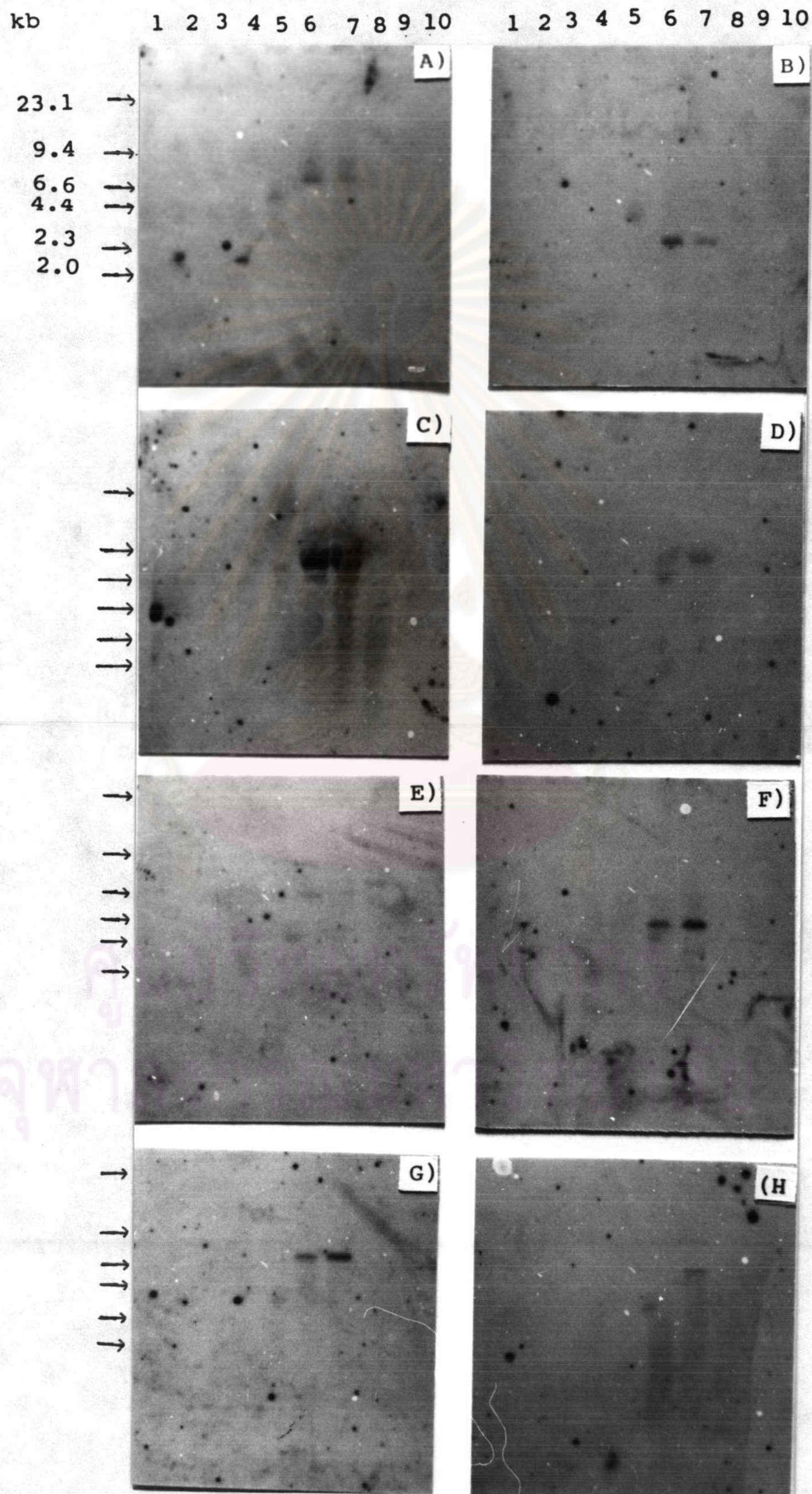

Table 3.3. RFLP of *nodC* gene hybridized to 10 strains of N_2-fixing bacteria

Strain	Size of restriction fragment (kb)							
	A: *BamHI*	B: *BglII*	C: *EcoRI*	D: *HindIII*	E: *PstI*	F: *SalI*	G: *SmaI*	H: *XhoI*
R15, R17, MG13	-	-	-	-	-	-	-	-
M5a1	-	-	-	-	-	-	-	-
Sp7	-	-	-	-	-	-	-	-
TAL380	8.3	3.9	9.0,6.5	9.4	9.0,5.5	6.0,4.5,3.7	9.0	-
TAL1372	8.3	3.9	9.0,6.5	9.4	5.6	6.0,4.5,3.7	9.0	6.8
TAL1402	-	-	-	-	6.3	-	-	-
THA5	-	-	-	-	-	-	-	-
THA7	-	-	-	-	-	-	-	-

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than *R. meliloti* TAL380 and TAL1372, no detectable DNA fragment was observed. Southern hybridization of *nif*HDK labeled probe (Figure 3.12. and Table 3.4.) with the 3 μg chromosomal DNA digested with 8 restriction enzymes shows similar RFLP patterns of the free-living *K. pneumoniae* M5a1 and the associative *K. oxytoca* strain R15, R17 and NG13 in *Bam*HI, *Eco*RI, *Hind*III, *Sal*I and *Xho*I cuttings. Different RFLP patterns between the free-living and associative *Klebsiella* have been observed with *Bgl*II, *Pst*I and *sma*I cuttings. These results indicate that the restriction patterns of the *nif* structural genes region of these associative *K. oxytoca* strains are very similar to *K. pneumoniae* M5a1, the free-living diazotroph except some different base sequences recognized by *Bgl*II, *Pst*I and *Sma*I. However, there were no difference detected among the three associative *K. oxytoca* strain R15, R17 and NG13 when hybridized with any *nod* or *nif* genes probes. All symbiotic *R. meliloti*, *R. leguminosarum* and *B. japonicum* and *A. brasilense* have shown no distinguished band of sequence homology with the *K. pneumoniae nif* structural genes in 3 μg of their chromosomal DNA. Only faint bands were observed in *B. japonicum* THA5 and THA7 digested with *Bam*HI, *Eco*RI, *Sma*I, *Sal*I and *Xho*I.

probe: *nif*HDK

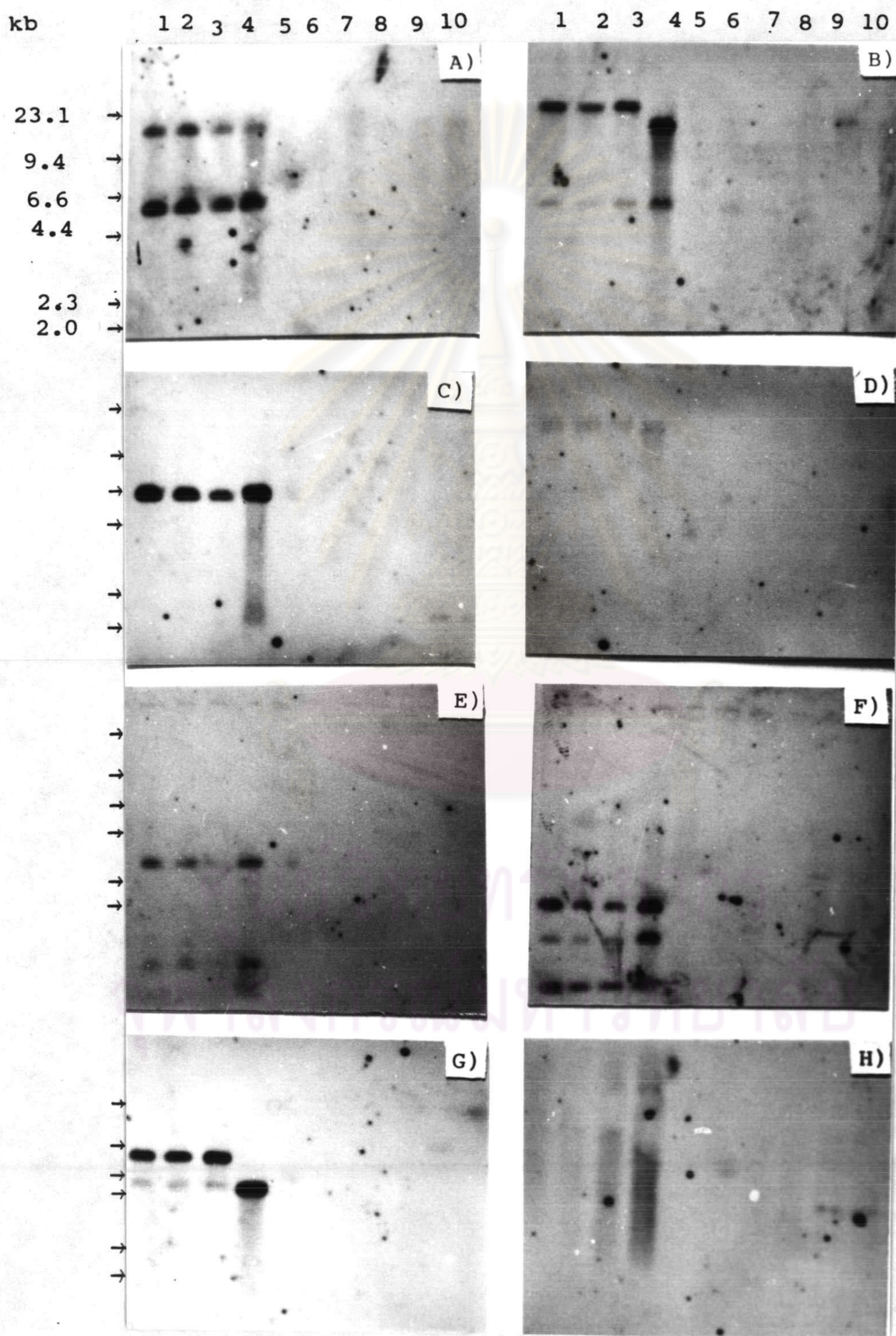

Table 3.4. RFLP of *nif*HDK gene hybridized to 10 strains of N_2-fixing bacteria

Strain	Size of restriction fragment (kb)							
	A: *Bam*HI	B: *Bgl*II	C: *Eco*RI	D: *Hind*III	E: *Pst*I	F: *Sal*I	G: *Sma*I	H: *Xho*I
R15, R17, MG13	20.0,5.2	23.4,3.9	6.2	21.0,16.5	3.4,1.5,1.2,1.0	3.5,2.4,1.4	9.6,5.8	-
M5a1	20.0,5.2	14.0,3.9	6.2	21.0,16.5	3.4,1.2,1.0	3.5,2.4,1.4	5.8	-
Sp7	-	-	-	-	-	-	-	-
TAL380	-	3.9	-	-	-	6.0	-	-
TAL1372	-	3.9	-	-	-	-	-	-
TAL1402	-	3.9	-	-	-	-	-	-
THA5	23.4,17.0	14.0	0.56	-	-	5.0	18.0	5.0
THA7	23.4,17.0	-	0.56	-	-	5.0	18.0	5.0

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3.5. Cloning of 4.0-4.9 kb fragments of chromosomal DNA from *K. oxytoca* NG13 into *Bam*HI site of pUC18

*Bam*HI fragment of pUC18 (2.7 kb) and chromosomal DNA from *K. oxytoca* NG13 (4.0-4.9 kb) were ligated in the ratio 1:5 (W/W), the products of ligation were transformed into *E. coli* JM101 and selected white colonies on LB agar plate containing ampicillin 50 μg/ml, X-gal 20 μg/ml and IPTG 25 μg/ml. There were 11 recombinant plasmids which form white colonies showing Ap^r and lac Z^-. Plasmid DNA isolated from these 11 clones were analyzed by agarose gel electrophoresis (Figure 3.13.) to check for the 4.0-4.9 kb fragments of chromosomal DNA from *K. oxytoca* NG13 insert. Dot hybridization with *nod*D1 probe shows positive dot hybridization on recombinant plasmid R1-R5, and R10 (Figure 3.14.). Only recombinant plasmids R1 and R2 were digested with *Bam*HI and analysed by 0.7% agarose gel electrophoresis. The size of inserted DNA, were approximately 4.0 and 4.9 kb and also hybridizable with *nod*D1 (Figure 3.15.) indicating that *nod*D1-liked genes have been cloned from *K. oxytoca* NG13.

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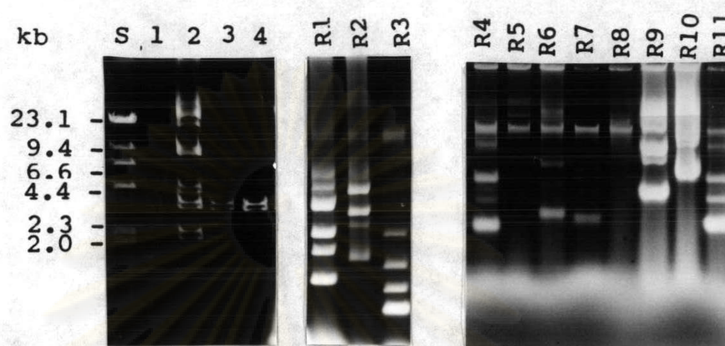

Figure 3.13. Electrophorogram of recombinant plasmids: R1-R11 after ligation between *Bam*HI fragment of pUC18 and *Bam*HI digested chromosomal DNA from *K. oxytoca* NG13

lane S : λ/*Hind*III

lane 1 : *K. oxytoca* NG13/*Bam*HI (4.0-4.9 kb)

lane 2 : pUC18

lane 3-4: pUC18/*Bam*HI (2.7 kb)

lane R1-R11: recombinant plasmids after digestion with *Bam*HI

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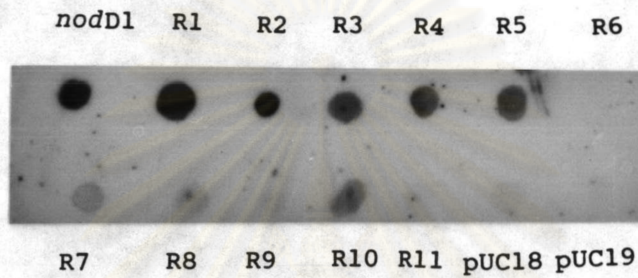

Figure 3.14. Dot hybridization between labeled *nod*D1 probe and recombinant plasmids: R1-R11 1.0 μg comparing with nonlabeled *nod*D1 100 ng and vectors 100 ng

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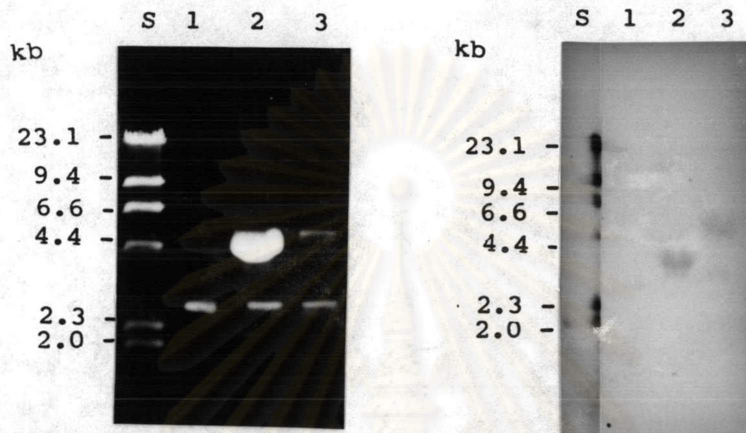

Figure 3.15. Southern hybridization between *nod*D1 probe and DNA from recombinant plasmids: R1 and R2 digested with *Bam*HI

The recombinant plasmids 2.0 μg were digested with *Bam*HI and hybridized with DIG-*nod*D1 probe (amount of 30 ng/ml). The hybridization was performed at 65-68 °C for 24 hr. The membrane was exposed to X-ray film at 37 °C for 30 min with intensifying screens.

lane S : λ/*Hind*III

lane 1 : pUC18 (2.7 kb)

lane 2 : DNA from recombinant plasmid R1

lane 3 : DNA from recombinant plasmid R2