CHAPTER III

RESULTS

Physical properties of strains used

Azotobacter spp.

To ascertain the right taxonomic classification of strains used, some growth properties of bacteria were verified, especially their specific capability to utilize certain types of organic compounds as the sole carbon source . As summarized in Table 4, they were not auxotrophs because all strains could grow well in Burk's nitrogen-free medium. Interestingly, different ionic strengths of rich media could be a certain extent devide Azotobacter into two groups. A. vinelandii KT1 and A. paspali B should be complied in the same group, since they prefered rich medium of higher ionic strength, therefore they growed better in LB than RM. Whereas the rest, should belong to the second group, perfered rich medium of lower ionic strength, therefore they multiplied better in RM than to LB. Furthermore, all strains showed their specific natures to use mannitol, rhamnose and starch as the sole carbon sources under nitrogen fixing condition. As judged by an observable turbidity of a culture ; A. paspali B failed to grow at all conditions mentioned whereas A.vinelandii gave cultures of visible cloudy in media having mannitol and rhamnose as the sole carbon sources. Azotobacter spp. NP also showed a similarity to Azotobacter chroococcum KT, then it should be classified into A. chroococcum. A. vinelandii KT2 showed clearly

that it was not similar to A. vinelandii KT1 because growth patterns revealed very quite different. In short, all of them seemed to be appropriate strains for our further studies

Azospirillum spp.

Veiwed from the oxygen content required for growth, Azotobacter is classified as the obligatory areobic type whereas Azospirillum is only a semiaerophile. However, veiwed from ability to multiply in an higher ionic strength medium, Azotobacter showed more sensitivity than that of Azcspirillum as seen in the Table 5. Again, all strains used were not auxotrophs, since they all multiplied in nitrogen-free medium eventhough of different fixed turbidity.

An investigation of the presence of plasmid

Three conventional methods for a determination of a presence of plasmid in bacterium had been performed.

1. Modified Eckhardt lysate electrophoresis

The protocol of this method is considered most gentle compared to those of the others. In principle, spheroplasts are prepared and are further lysate by SDS in gel slot at a constant voltage of 15 volt, the covalently closed circular plasmids then migrate through the pore of agarose gel without facing any drastic condition. That means the observedplasmids should be the intact forms of the plasmid.

All strains of being previously classified were analysed and the results obtained were summarized in figure 4. All Azotobacter spp. showed only the upper fuzzy band, which migrated over the 23.5 kb fragment of Hind III digested λ-DNA (figure 4A). However, the

Table 4. The growth properties of Azotobacter spp.

Bacteria	agar plate[1]			Burk's(N-free)+carbohydrate[2]		
	LB	Burk's(N-free)	RM	mannitol	rhamnose	starch
A.vinelandii KT1	+++	+	++	+	+	-
A.vinelandii KT2	-	+++	+++	++	++	-
A.chroococcum KT	-	+++	+++	+++	-	+++
Azotobacter spp.NP	-	+++	+++	++	-	+++
A.paspali B	+++	+	++	-	-	-

1 A single colony of bacteria was streaked on agar plate medium and then incubated at 30°C for 3 days.

2 A single colony of bacteria was inoculated into 25 ml Erlenmeyer flask containing 7 ml of Burk's medium which has 1 % of rhamnose or mannitol as a sole carbon source and bromothymol blue as a pH indicator. The culture was incubated on rotary shaker at 30°C for 3 days. Growth was observed from the change of color from blue to yellow. The ability to use starch as a sole carbon source was tested by streak bacteria on Burk's agar plate containing 1 % of soluble starch, the colony was observed after incubation at 30°C for 3 days.

- \- no growth or colonies size almost invisible with naked eyes.
- \+ slightly grow
- ++ grow well
- +++ grow very well

Table 5. The growth properties of Azospirillum spp.

Bacteria	LB agar plate (1)	NF semi-solid medium (2)
Azospirillum brasilense Sp7	+++	+++
Azospirillum brasilense A2	+++	+
Azospirillum lipoferum SpMRAI	+++	+
Azospirillum spp. A12	+++	+++

(1) : A single colony of bacteria was streaked on agar plate and then incubated at 30°C for 2 days.

(2) : One mililitre of starter culture was inoculated into 3x20 cm. test tube containing 30 ml of NF semi-solid medium. The culture was incubated in 30°C without shaking for 3 day.

reference strains, Escherichia coli HB101 (pBR322) and HB101 (containing pSA30) gave also the same level of fuzzy band (lane 2,3 and 4). Therefore, they should be interpreted as sheared chromosomal DNA or sheared chromosomal DNA comigrated with large plasmid. This results were found consistent eventhough the electrophoresis had been performed in a very long period of time.

In case of Azospirillum, there were three strains that gave some extra bands in addition of those fuzzy bands, the similar bands to those of Azotobacter spp. (Figure 4B). Therefore, these extra sharp bands should be interpreted as plasmid bands. The molecular size of the Azospirillum plasmid was estimated by comparing the mobility to the standard plasmids, pBR322 (4.36 kb), pSA30 (10.9 kb) and pRD1 (86 kb). Four bands of plasmid were detected in Azospirillum spp. A12 (Figure 4B and 4C), three dominated bands which migrated near the band of pRD1 represented the high molecular weight plasmid, approximately 70, 80 and 90 kb in size, and one very weak band which migrated near the 9.7 kb fragment of λ/HindIII and pSA30 plasmic represented the small plasmid, approximately 11 kb in size. Two bands of plasmid were found in A. brasilense Sp7 (lane 8 in figure 4B), the molecular sizes were approximately 75 and 100 kb. Whereas A. lipoferum SpMRA1 contained one small plasmid approximately 9 kb in size. In A. brasilense A2, any bands of plasmid could not be found. Although period of electrophoresis were varied, no plasmid could be detected in these strains of Azospirillum spp.

Figure 4. Ethidium bromide staining patterns of plasmid profiles of five strains of Azotobacter and four strains of Azospirillum by Modified Eckhardt lysate electrophoresis.

A. The gel was 0.6 % agarose and electrophoresis was performed at constant voltage of 15 volt for 2 h, then at 80 volt for 3 h.

lane 1, λ -DNA digested with Hind III as a DNA marker

lane 2, E.coli HB101 (pBR322)

lane 3, E.coli HB101 (pSA30)

lane 4, E.coli K12 JC5466 (pRD1)

lane 5, Azotobacter vinelandii KT1

lane 6, Azotobacter vinelandii KT2

lane 7, Azotobacter chroococcum KT

lane 8, Azotobacter paspali B

lane 9, Azotobacter chroococcum NP

B. The gel was 0.6 % agarose and electrophoresis was performed at constant voltage of 15 volt for 2 h, then at 80 volt for $4\frac{1}{2}$ h.

lane 1, λ /Hind III as a DNA marker

lane 2, E.coli HB 101 (pSA30)

lane 3, E.coli C 600

lane 4, E.coli K12 JC5466 (pRD1)

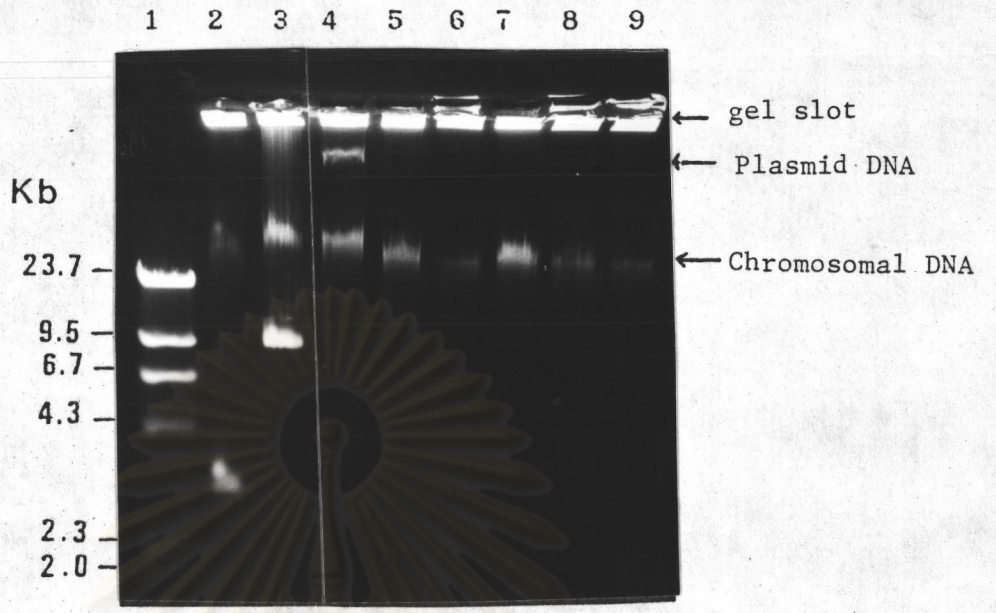
lane 5, Azospirillum lipoferum SpMRA1

lane 6, Azospirillum brasilense A2

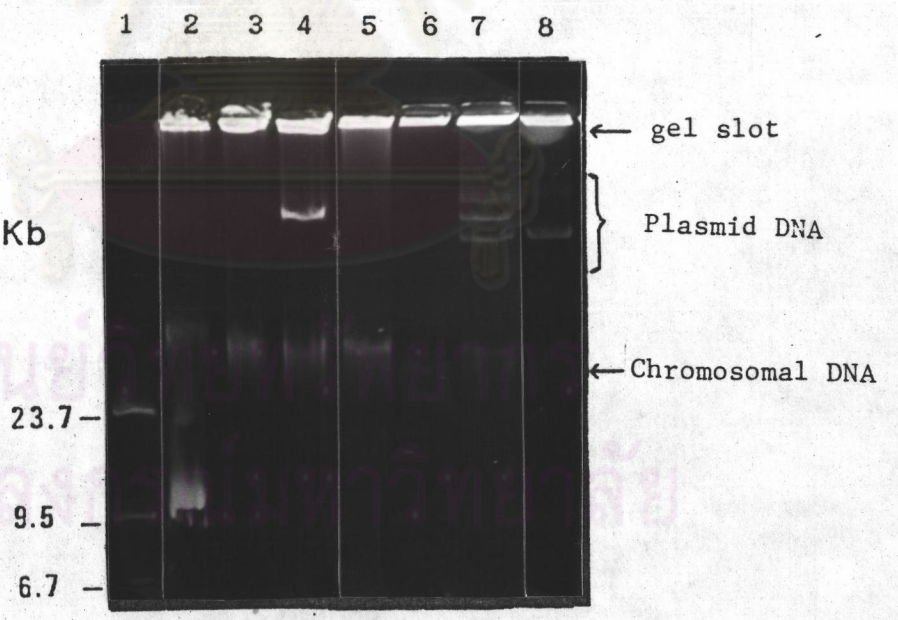
lane 7, Azospirillum spp. A12

lane 8, Azospirillum brasilense Sp7

A


1 2 3 4 5 6 7 8 9
gel slot
Plasmid DNA
Kb
Chromosomal DNA
23.7
9.5
6.7
4.3
2.3
2.0

B


1 2 3 4 5 6 7 8
gel slot
Kb
Plasmid DNA
Chromosomal DNA
23.7
9.5
6.7

C. The gel was 0.6 % agarose and electrophoresis was performed at constant voltage of 15 volt for 2 h, then at 80 volt for 4 h.

lane 1, λ /Hind III

lane 2, E.coli HB101 (pBR322)

lane 3, E.coli HB101 (pSA30)

lane 4, E.coli K12 JC5466 (pRD1)

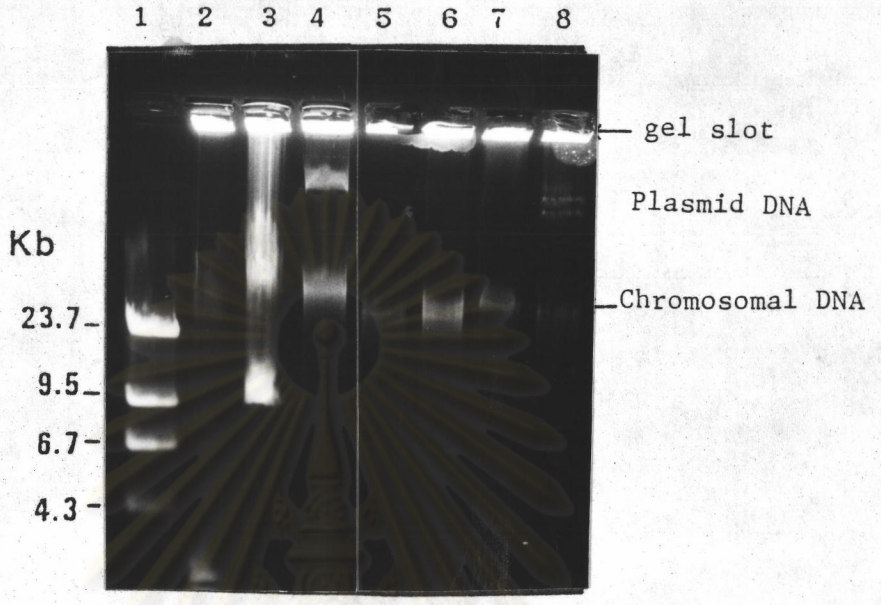
lane 5, E.coli C 600

lane 6, Azospirillum brasilense A2

lane 7, Azospirillum lipoferum SpMRA1

lane 8, Azospirillum spp. A12

C



2. Large plasmid isolation's method (Hensen and Olsen, 1978)

This method requires more reagents and time when compared to that of the modified Eckhardt's method. The principle of the method is spheroplasts that are generated after lysozyme treatment will be lysed by SDS and heat treatment. The extracted plasmid analyzed are shown in figure 5, all Azotobacter spp (lane 6-10) showed only the upper fuzzy band, represented the sheared chromosomal DNA. The lower two molecular weight bands might be ribosomal RNA which contaminated in the preparation because they could be eliminated by digestion with RNase (data not shown). No bands of plasmid was found in five strains of Azotobacter spp.

In case of Azospirillum, the results are similar to those obtained from the first method. But in the case of Azospirillum spp. A12 and A. brasilense Sp7, the largest plasmid found from the first method could not be clearly demonstrated in this experiment. Although, the amount of plasmid DNA sample was increased three times, the high smear background was observed (data not shown).

3. Rapid alkaline extraction

The last method of plasmid detection was rapid alkaline extraction. This method requires lysozyme to generate spheroplast and lysis of the spheroplast by SDS in alkaline condition. The chromosomal DNA and the plasmid are denatured by alkali in this step. Upon neutralization with sodium acetate, pH 4.8, the chromosomal DNA forms an insoluble net work while the plasmid is remained double-stranded.

The contents of plasmid of Azotobacter spp. could be confirmed by using this method. Plasmid profiles are shown in figure 6. The

results are identical to those obtained from the previous methods, no plasmid band was detected in all strains of Azotobacter spp. However, plasmid bands were observed in E. coli which harbours plasmid (lane 2-4). The yeild of extracted plasmid from this method was higher than the others (60-100 ng/ml culture) and only small amount of chromosomal DNA and ribosomal RNA were present in this preparation. This method is seemed to be the suitable method for large scale preparation of plasmid.

The plasmid content of nine bacterial strains of Azotobacter spp. and Azospirillum spp. are summarized in Table 6.

Preparation of chromosomal DNA and digestion with restriction endonuclease

Chromosomal DNA were extracted from nine strains of nitrogen fixing bacteria using modified method of Rodriquez (1983). The modification was only that the ethanol precipitation was performed in the presence of 2.5 M. ammonium acetate instead of 0.2 M. NaCl. The extracted DNA was free from protein and RNA since the ratio of OD_{260}/OD_{280} of various samples of extracted DNA was always in the range of 1.8-1.9. Furthermore, the extracted DNA was in the form of high molecular weight DNA since RNA and small fragmented DNA were not present in this preparation. Very sharp band was obtained as analyzed by agarose gel electrophoresis (Figure 7).

From the study of restriction endonuclease digestion of Azotobacter chromosomal DNA, it was found that chromosomal DNA extracted from Azotobacter which were grown in Burk's N-free medium could not be cut by every test of restriction endonucleases. But, when Azotobacter were grown in rich medium (RM), the isolated

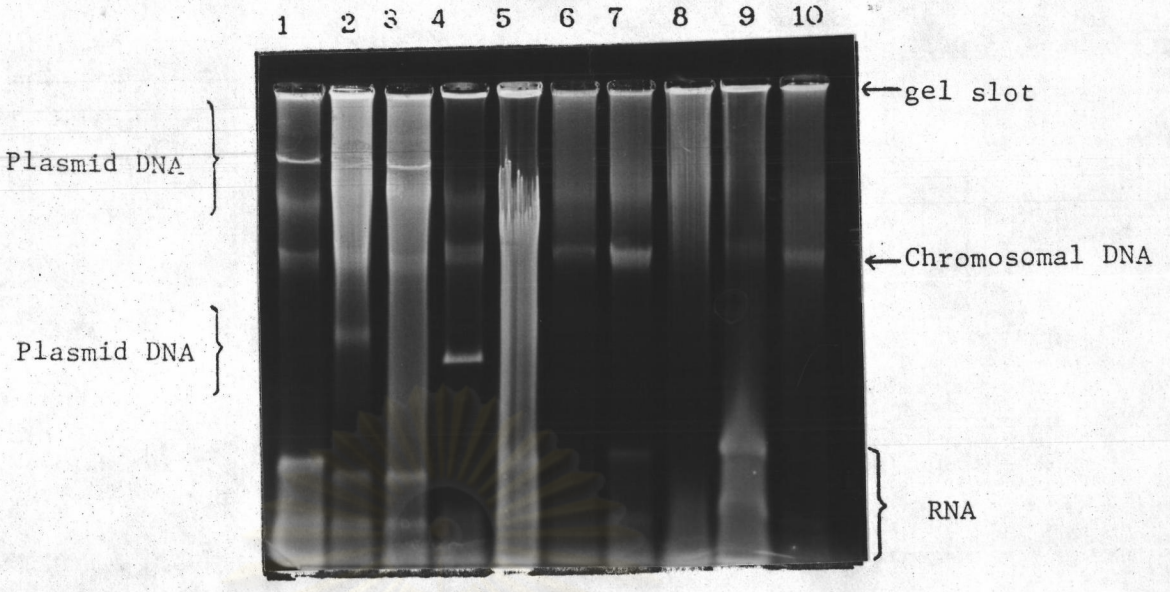


Figure 5. Ethidium bromide staining patterns of plasmid profile extracted from various species and strains of Azotobacter and Azospirillum which were prepared by method of Hansen and Olsen (1978).

The extracted plasmid DNA (400 ng) were loaded on 0.7 % agarose gel and electrophoresis in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, plasmid pRD1

lane 2, Azospirillum spp. A12

lane 3, Azospirillum brasilense Sp7

lane 4, Azospirillum lipoferum SpMRA1

lane 5, Azospirillum brasilense A2

lane 6, Azotobacter vinelandii KT1

lane 7, Azotobacter vinelandii KT2

lane 8, Azotobacter chroococcum KT

lane 9, Azotobacter paspali B

lane 10, Azotobacter chroococcum NP

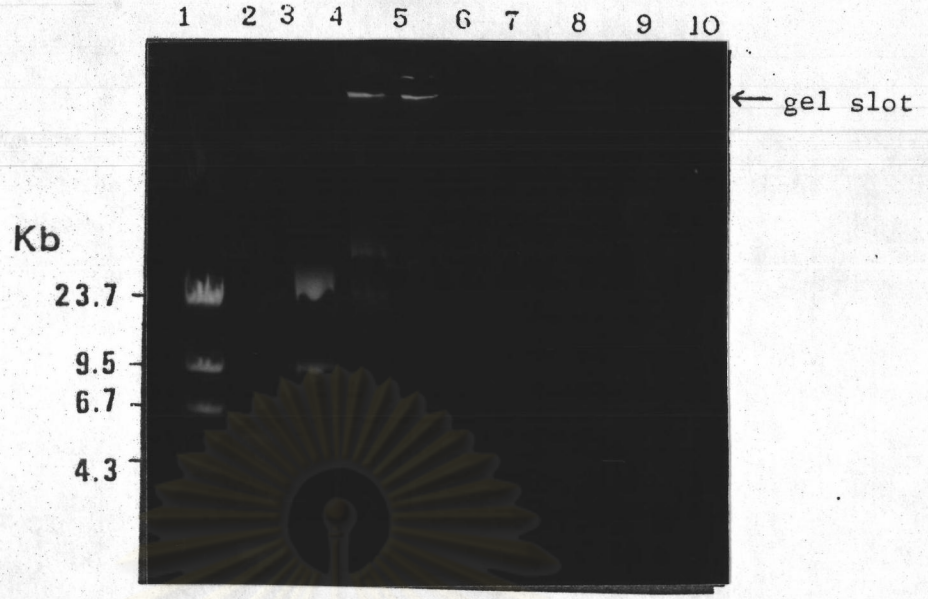


Figure 6. Ethidium bromide staining patterns of plasmid profile extracted from various species and strains of Azotobacter by rapid alkaline extraction procedure.

The extracted plasmid DNA were loaded on 0.7 % agarose gel and electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph was taken under UV light.

lane 1, λ -DNA digested with Hind III
lane 2, pBR322
lane 3, pSA30
lane 4, pCK3
lane 5, E.coli C 600
lane 6, Azotobacter vinelandii KT1
lane 7, Azotobacter vinelandii KT2
lane 8, Azotobacter chroococcum KT
lane 9, Azotobacter paspali B
lane 10, Azotobacter chroococcum NP

Table 6. Plasmids in Azotobacter spp. and Azospirillum spp. : number and sizes

Strains	Number of plasmids	Plasmid size (approximate in kb)
Azotobacter vinelandii KT1	-	-
Azotobacter vinelandii KT2	-	-
Azotobacter chroococcum KT	-	-
Azotobacter chroococcum NP	-	-
Azotobacter paspali B	-	-
Azospirillum brasilense Sp7	2	75,100
Azospirillum lipoferum SpMRAI	1	9
Azospirillum brasilense A2	-	-
Azospirillum spp. A12	4	11, 70, 80, 90

note :-, not found any plasmid.

chromosomal DNA could be cut by restriction endonuclease, However, Azotobacter and Azospirillum chromosomal DNA extracted according to the method of Rodriquez (1983) can not be completly cut by restriction endonuclease. The digestion could not be improved eventhough as high as 10 units of restriction enzymes were used per 1 ug of extracted chromosomal DNA. The chromosomal DNA extracted from the same organism using modified method of Rodriquez (1983) in which ethanol precipitation was performed in the presence of 2.5 M ammonium acetate instead of 0.2 M NaCl were completely digested with restriction endonuclease. The example of the digestion was shown in figure 8. From this result clearly showed that ethanol precipitation of DNA in the presence of 2.5 M ammonium acetate was more effecting for eliminating the inhibitors of restriction endonuclease digestions from precipitated DNA. These inhibitors may be detergents and other undefined contaminate.

Various strains of Azotobacter and Azospirillum were digested with suitable amount of restriction endonuclease. The suitable smount of restriction enzymes are the smallest amount of enzyme that digests the chromosomal DNA into complete (as analyzes by the constant reproduce restriction pattern upon the addition of the enzyme). Ethidium bromine banding patterns of extracted chromosomal DNA from various species and strains of Azotobacter and Azospirillum after digestion with SmaI, BglII, EcoRI, PstI, BamHI, HindIII and HaeIII are shown in figure 9-17. After digestion, DNA fragments with heterogeneous molecular sizes were obtained. SmaI, and EcoRI digestion of Azotobacter and Azosipirillum DNA generated wide range of fragment sizes (Figure 9 and 10). Moreover, the digestion patterns among various species and strains of Azotobacter and Azospirillum

show many differences.

Mainly large fragments were obtained by digestion of Azotobacter and Azospirillum DNA with BglII (Figure 11). Most strains gave similar but not identical restriction patterns. Surprisingly, Azospirillum brasilense A2 DNA could not be cut by BglII (lane 10). Digestion patterns show many difference among various species and strains of Azotabacter and Azospirillum after digestion of their chromosomal DNA with PstI (figure 12 and 13). Small fragments (< 10 kb in size) were obtained from Azotobacter paspali B chromosomal DNA digestion (Figure 12, lane 5). Ethidium bromide banding patterns of extracted chromosomal DNA from various species and strains of Azotobacter and Azospirillum after digestion with BamHI or HindIII showed many differences. Wide range of fragment sizes were obtained (figure 14, 15 and 16).

When chromosomal DNA of Azotobacter or Azospirillum DNA were digested with HaeIII, which is four-base recognition endonuclease (Figure 17). Mainly small fragments (< 1 kb) were obtained. Two strains of Azospirillum, SpMRA1 and A2 (lane 9 and 10), gave the larger HaeIII - digested fragments than the others.

Preparation and characterization of pSA30

As mentioned earlier, the recombinant plasmid pSA30 was constructed by insertion of nif structural genes from Klebsiella pneumoniae at EcoRI site of pACYC184 and the transformants containing pSA30 were tetracycline resistance (Cannon et al., 1979). In this study, pSA30 was used as a DNA probe in hybridization experiments. Therefore, large scale preparation of pSA30 was required. Prior to

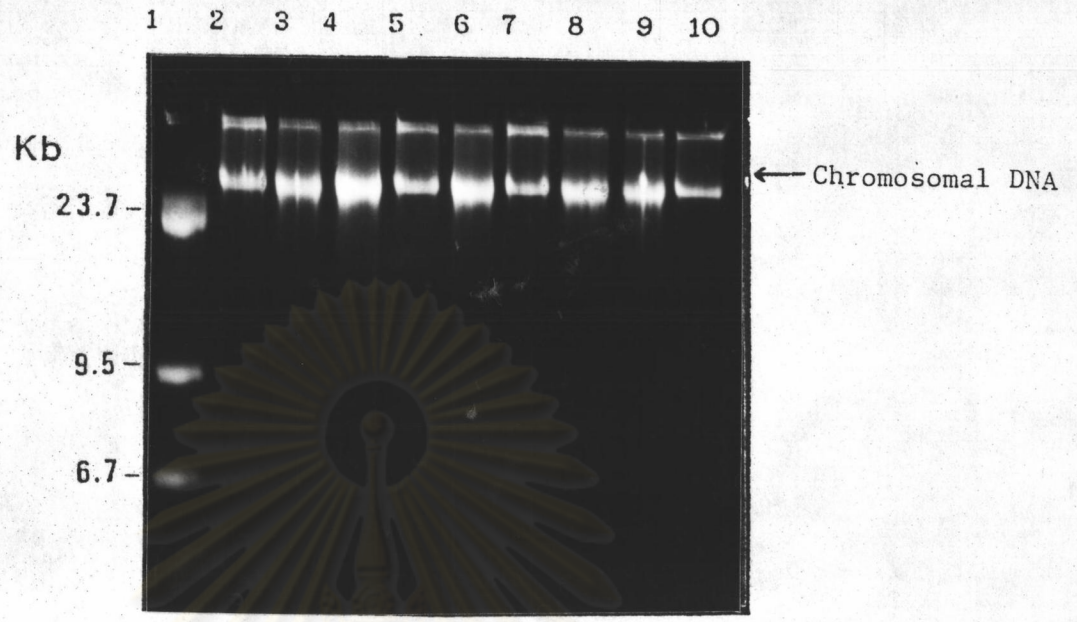


Figure 7. Ethidium bromide staining patterns of extracted chromosomal DNA from various species and strains of Azotobacter and Azospirillum.

One microgram of extracted DNA was loaded in each well of 0.7 % agarose gel and electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph of the gel was taken under UV light.

lane 1, 500 ng of λ -DNA digested with Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum lipoferum SpMRAI
lane 9, Azospirillum brasilense A2
lane 10, Azospirillum spp. A12

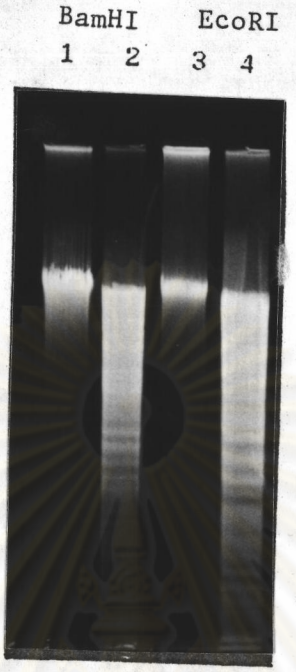


Figure 8. Ethidium bromide staining pattern of chromosomal DNA digested with restriction endonuclease on agarose gel.

Digestion of chromosomal DNA of Azotobacter vinelandii KT1 which prepared by the method of Rodriquez (1983) and the modified method as ammonium acetate precipitation were loaded on 0.7 % agarose gel,then electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, Bam HI digested DNA which prepared by Rodriquez's method
lane 2, Bam HI digested DNA which prepared by the modified Rodroquez's mothod.
lane 3, EcoRI digested DNA which prepared by Rodriquez's method
lane 4, EcoRI digested DNA which prepared by the modified Rodriquez's method.

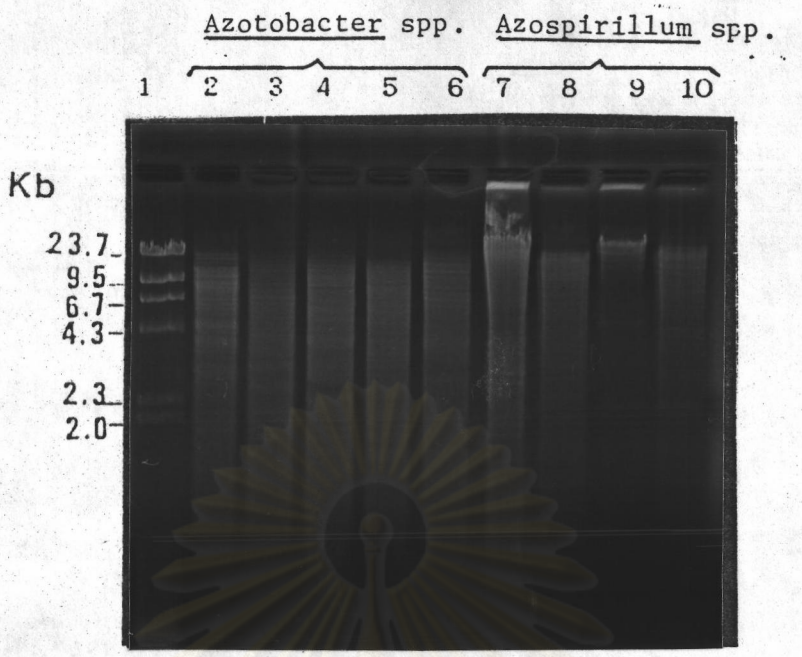


Figure 9. Ethidium bromide staining patterns of SmaI digested fragments of chromosomal DNA from five strains of Azotobacter and four strains of Azospirillum.

Four microgram of chromosomal DNA were digested with 50 units of SmaI and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light..

lane 1, λ -DNA digested with Hind III, as a size marker
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRA1
lane 10, Azospirillum brasilense A2

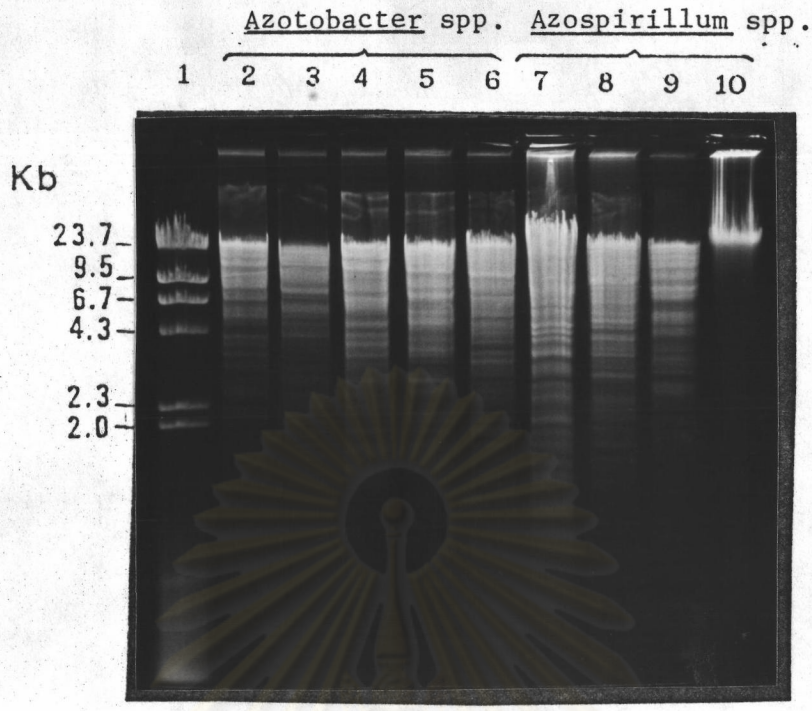


Figure 10. Ethidium bromide staining patterns of BglII digested fragments of chromosomal DNA from five strains of Azotobacter and four strains of Azospirillum.

Four microgram of chromosomal DNA were digested with 40 units of Bgl II and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRA1
lane 10, Azpspirillum brasilense A2

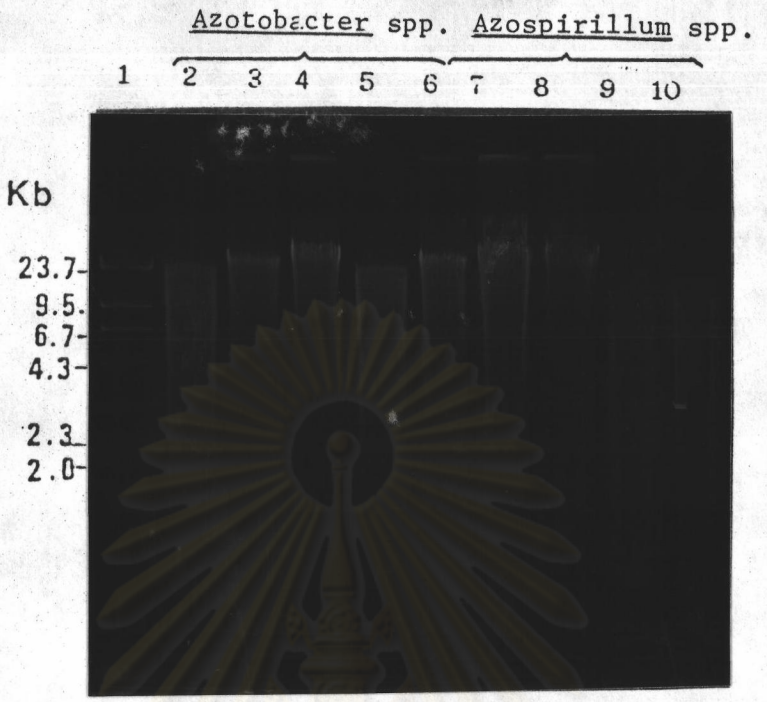


Figure 11. Ethidium bromide staining patterns of EcoRI digested fragments of chromosomal DNA from Azotobacter spp. and Azospirillum spp.

Six microgram of chromosomal DNA were digested with 100 units of EcoRI and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRA1
lane 10, Azospirillum brasilense A2

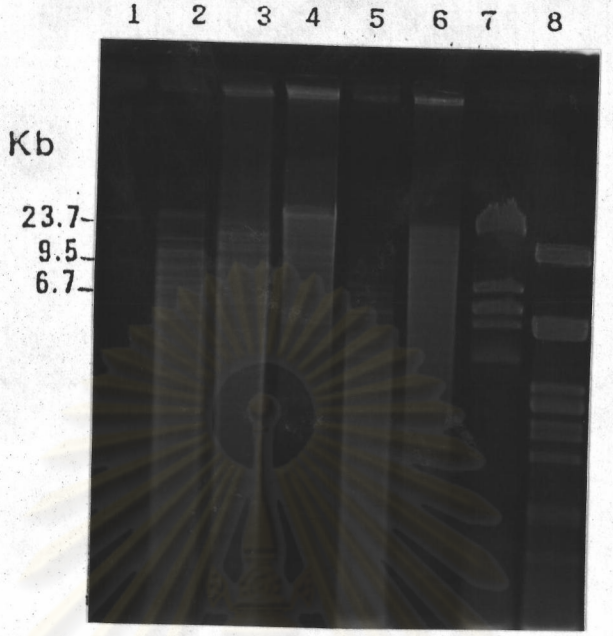


Figure 12. Ethidium bromide staining patterns of Pst I digested fragments of chromosomal DNA from five strains of Azotobacter on agarose gel.

Four microgram of chromosomal DNA were digested with 80 units Pst I and then were loaded on 0.7 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph of the gel was taken under UV light.

lane 1, λ /Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP.
lane 7, λ /EcoRI
lane 8, λ /Pst I

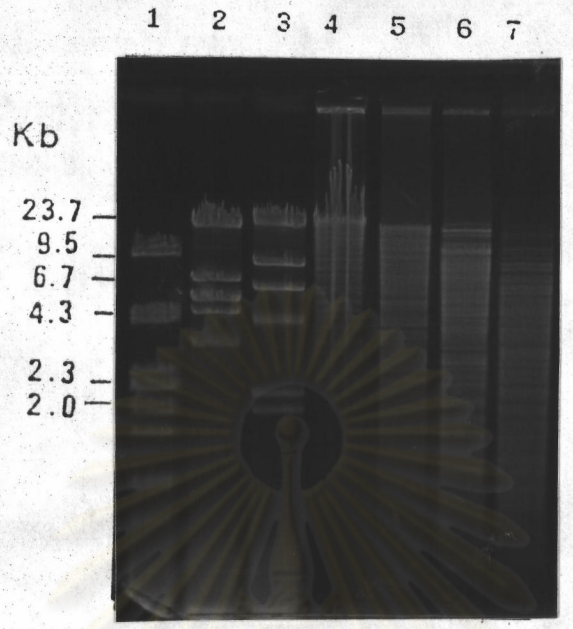


Figure 13. Ethidium bromide staining patterns of Pst I degested fragments of chromosomal DNA from four strains of Azospirillum on agarose gel.

Four microgram of chromosomal DNA were digested with 80 units of Pst I and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Pst I
lane 2, λ /EcoRI
lane 3, λ /Hind III
lane 4, Azospirillum lipoferum SpMRA1
lane 5, Azospirillum brasilense Sp7
lane 6, Azospirillum brasilense A2
lane 7, Azospirillum spp. A12

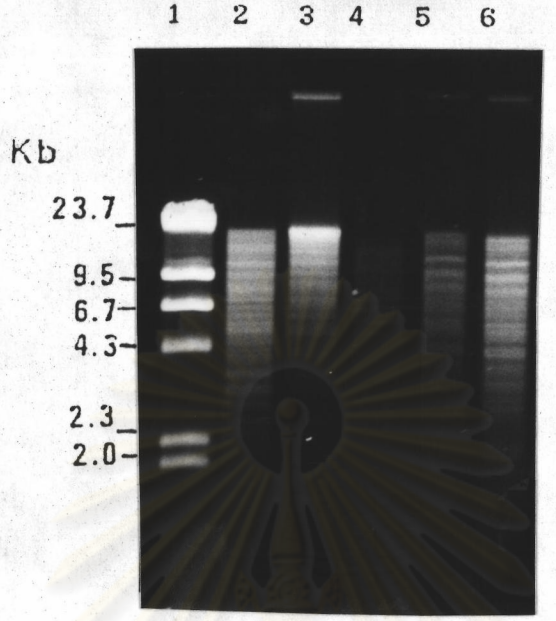


Figure 14. Ethidium bromide staining patterns of BamHI digested fragments of chromosomal DNA from five strain of Azotobacter.

Three microgram of chromosomal DNA were digested with 24 unit of BamHI and then were loaded on 0.7 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph of the gel was taken under UV light.

lane 1, Hind III digested fragments of λ DNA
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP

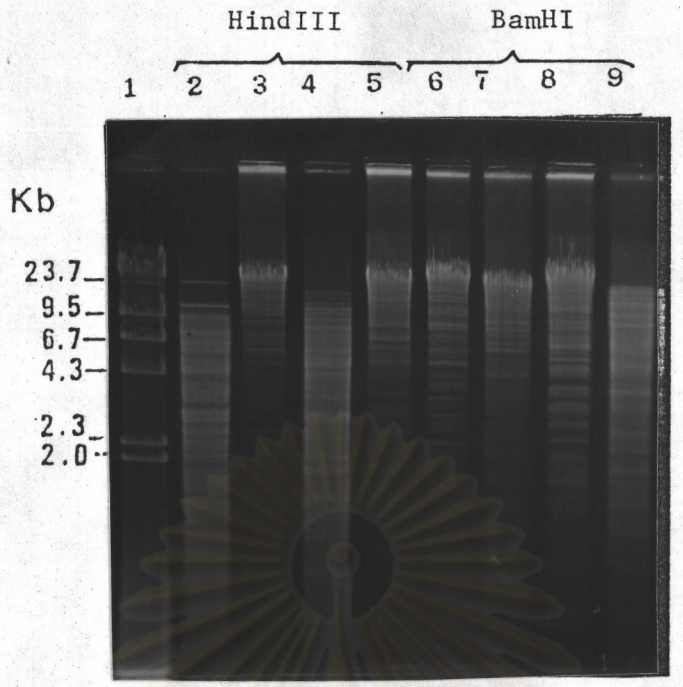


Figure 15. Ethidium bromide staining patterns of restriction endonuclease digested fragments of chromosomal DNA from four strains of Azospirillum on agarose gel.

Four microgram of chromosomal DNA were digested with 80 units of Bam HI or 250 units of Hind III, and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ -DNA digested with Hind III as a size marker
lane 2, Azospirillum lipoferum SpMRA1/Hind III
lane 3, Azospirillum brasilense Sp7/Hind III
lane 4, Azospirillum brosilense A2/Hind III
lane 5, Azospirillum spp. A12/Hind III
lane 6, Azospirillum lipoferum SpMRA1/Bam HI
lane 7, Azospirillum brasilense Sp7/Bam HI
lane 8, Azospirillum brasilense A2/Bam HI
lane 9, Azospirillum spp. A12/Bam HI

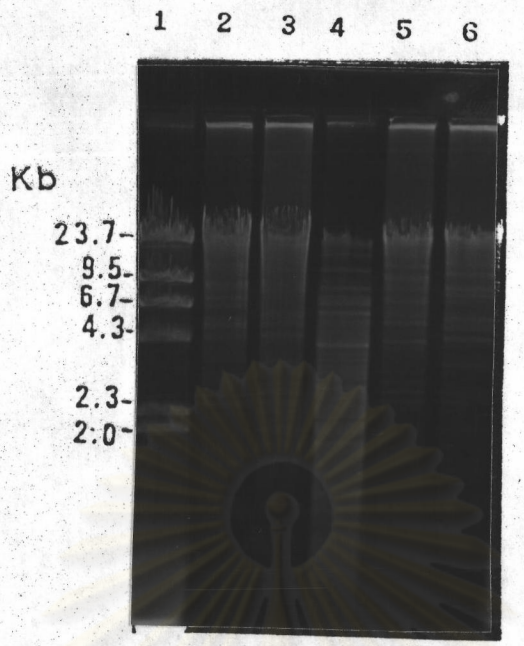


Figure 16. Ethidium bromide staining patterns of Hind III-digested fragments of chromosomal DNA from five strains of Azotobacter in agarose gel.

Four microgram of chromosomal DNA were digested with 250 units of Hind III and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ DNA digested with Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP

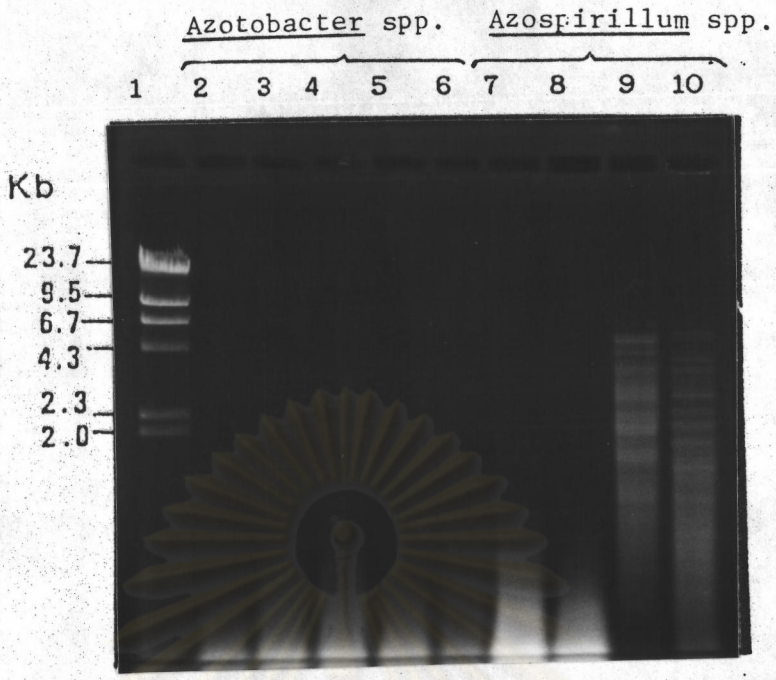


Figure 17. Ethidium bromide staining patterns of Hae III digested fragments of chromosomal DNA from Azotobacter spp. and Azospirillum spp.

Four microgram of chromosomal DNA were digested with 50 units of Hae III and then were loaded on 0.8 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRAI
lane 10, Azospirillum brasilense A2

plasmid purification, it was essential to characterize its restriction map that would be used as the marker of the preparation.

Large scale preparation of pSA30 was performed by rapid alkaline extraction from tetracycline resistant culture of E.coli HB101 (pSA30). The extracted plasmid was analyzed by agarose gel electrophoresis, Figure 18 clearly show that size of the extracted plasmid is larger than pBR322 (4.36 kb).

The restriction map of the suspected pSA30 was determined by digestion with BamHI, HindIII, EcoRI and SalI. The restriction fragments were resolved on 0.7 % agarose gel electrophoresis. According to the pSA30 restriction map reported by Cannon (1979) and Krol (1982) as shown in figure 2, 3.7 and 7.2 kb fragments would be obtained by BamHI digestion, 5.0 and 5.9 kb fragments must be obtained by HindIII digestion, 4.0 and 6.9 kb fragments would be obtained by EcoRI digestion whereas SalI digestion generates three linear DNA fragments of 1.6, 3.6 and 5.7 kb.

The result of digestion of the suspected pSA30 with BamHI, HindIII, EcoRI and SalI are shown in figure 19. The fragment sizes were calibrated using λ /Hind III fragments as a standard molecular size. The calibration curve is shown in Appendix 1. DNA fragments obtained from BamHI, HindIII, EcoRI or SalI of pSA30 corresponded to the expected fragments size calculated from the restriction map of pSA30. The results from this experiment clearly show that the extracted plasmid is pSA30.

Purification of pSA30 plasmid

Plasmid obtained from rapid alkaline extraction is always contaminated with residual small molecular weight RNAs sometimes with

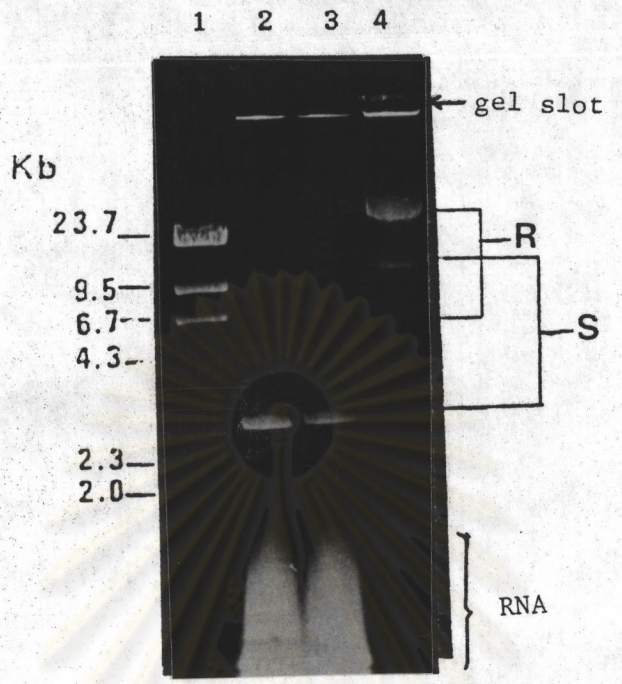


Figure 18. Ethidium bromide staining pattern of pSA30 plasmid extracted by rapid alkaline extraction procedure.

The extracted pSA30 plasmid was loaded on 0.7 % agarose gel and electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph was taken under UV light.

lane 1, λ digested with Hind III
lane 2, pBR322
lane 3, pBR322
lane 4, pSA30

S = supercoil form

R = relaxed form

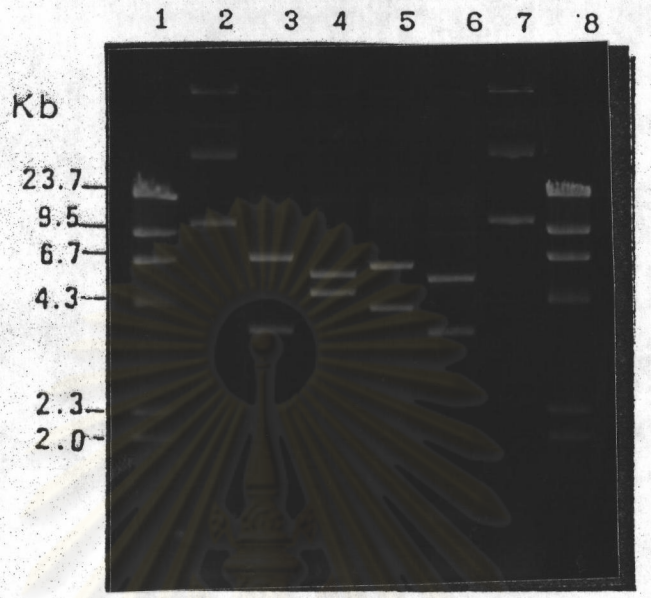


Figure 19. Ethidium bromide staining patterns of the plasmid and the restriction fragments on agarose gel.

Plasmid DNA and digested plasmid DNA were loaded on 0.7 % agarose gel and electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ DNA digested with Hind III as molecular weight size marker
lane 2, undigested pSA30
lane 3, Bam HI digested pSA30
lane 4, Hind III digested pSA30
lane 5, EcoRI digested pSA30
lane 6, Sal I digested pSA30
lane 7, undigested pSA30
lane 8, size marker of λ /Hind III fragments

high molecular weight DNA. Purification step is necessary for DNA which will be used as a probe in hybridization experiment.

The purification of the pSA30 was performed to recover only supercoiled plasmid using agarose gel. The purified supercoiled pSA30 is shown in lane 3 of figure 20. The recovery of supercoiled plasmid after this purification step was in the range of 20-30 %.

Preparation and purification of DNA fragments from pSA30

1. pACYC184 the DNA vector

After digestion of pSA30 with EcoRI, 4 kb fragment of lineared pACYC184 and 6.9 kb fragment of nif structural genes of Klebsiella pneumoniae were obtained. The ethidium bromide banding pattern of purified pACYC184 fragment is shown in lane 2 of figure 21.

2. The nif fragment A1 (part of nif K), nif fragment A2 (part of nif D) and nif fragment A3 (nif H and part of nif D).

According to the restriction map of pSA30 (figure 2 and 3), three linear molecules of nif fragment A1 (5.76 kb) which carries part of nif K, nif fragment A2 (1.44 kb) which carries part of nif D and nif fragment A3 (3.7 kb) which carries nif H and part of nif D from Klebsiella pneumoniae would be obtained after digestion of pSA30 with BamHI and Hind III. Then, the three fragments were purified by recovery from low-melting agarose gel as described in Materials and Methods. The ethidium bromide banding pattern of the purified fragments are shown in figure 22. The recovery of linear DNA fragments using this purification procedure (recovery from low-melting agarose gel) was in the range of 70-80 %.

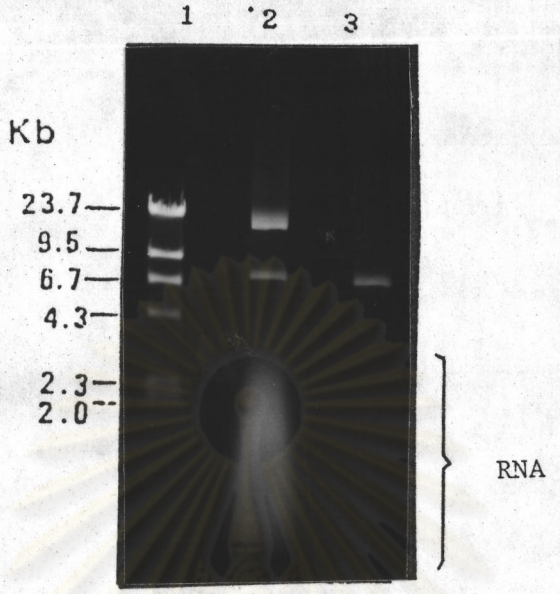


Figure 20. Ethidium bromide staining patterns of crude and purified supercoiled pSA30 plasmid on agarose gel.

The crude extracted and purified pSA30 were load on 0.7 % agarose gel and electrophoresed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph was taken under UV light.

lane 1, Hind III fragments of λ DNA
lane 2, 300 ng of crude extracted pSA30
lane 3, purified supercoiled pSA30

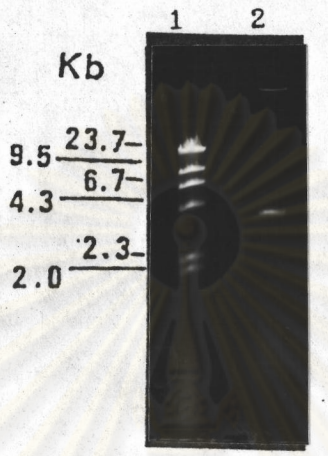


Figure 21. Ethidium bromide staining pattern of pACYC184 fragments.

After pSA30 was digested with EcoRI, the 4.0 kb fragments of pACYC184 part was purified by low-melting agarose gel then electrophoresed on 0.7 % agarose gel. After staining with ethidium bromide, the photograph of the gel was taken under UV light.

lane 1, λ DNA digested with Hind III
lane 2, pACYC184 fragment

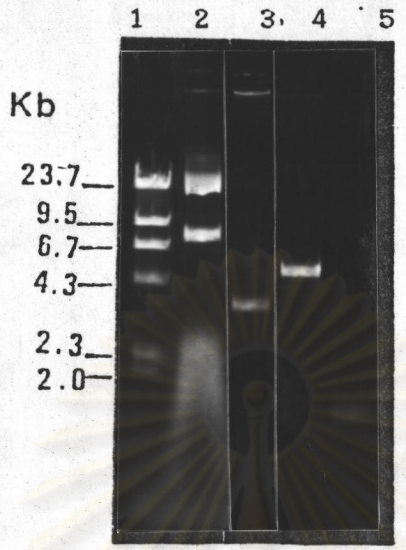


Figure 22. Ethidium bromide staining patterns of plasmid and purified nif fragments on agarose gel.

The several nif fragments were prepared from the pSA30. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph was taken under UV light.

lane 1, λ DNA digested with Hind III as a size marker
lane 2, crude pSA30
lane 3, purified nif fragment A3 (3.7-kb)
lane 4, purified nif fragment A1 (5.76-kb)
lane 5, purified nit fragment A2 (1.44-kb)

Labelling of DNA by nick translation

Plasmid and DNA fragments were labelled by nick translation using ^{32}P-dATP as described in Materials and Methods. An example of the kinetic of nick translation was shown in figure 23. The specific activity of the labelled probe obtained was 2-7x10^{7} cpm/ug DNA. Usually, the incroporation of the labelled nucleotide into purified pSA30 was about 40-50%, whereas for the linear DNA fragments were in the range of 50-80 %.

Characterization of the labelled plasmid

The labelled plasmids obtained from nick translation were confirmed to be pSA30 by comparing the electrophoretic mobility of the labelled plasmid with nonlabelled pSA30.

After electrophoresis of labelled plasmid on 1 % agarose gel, the gel was dried and exposed on X-ray film. The position of band on autoradiogram was compared to ethidium bromide banding pattern An example of characterization of labelled pSA30 is shown in figure 24. From the figure, the band on autoradiogram was found to be at the same position as the stained band of pSA30. This indicated that the labelled DNA was pSA30.

Localization of nif structural genes by nucleic acid hybridization

Principally, DNA-DNA hybridization will be used to verify the relatedness of particular organisms. Because, when mixed single-stranded DNA, carrying specific nucleotide sequences in common, will anneal at the regions of genetic homology to form duplexes.

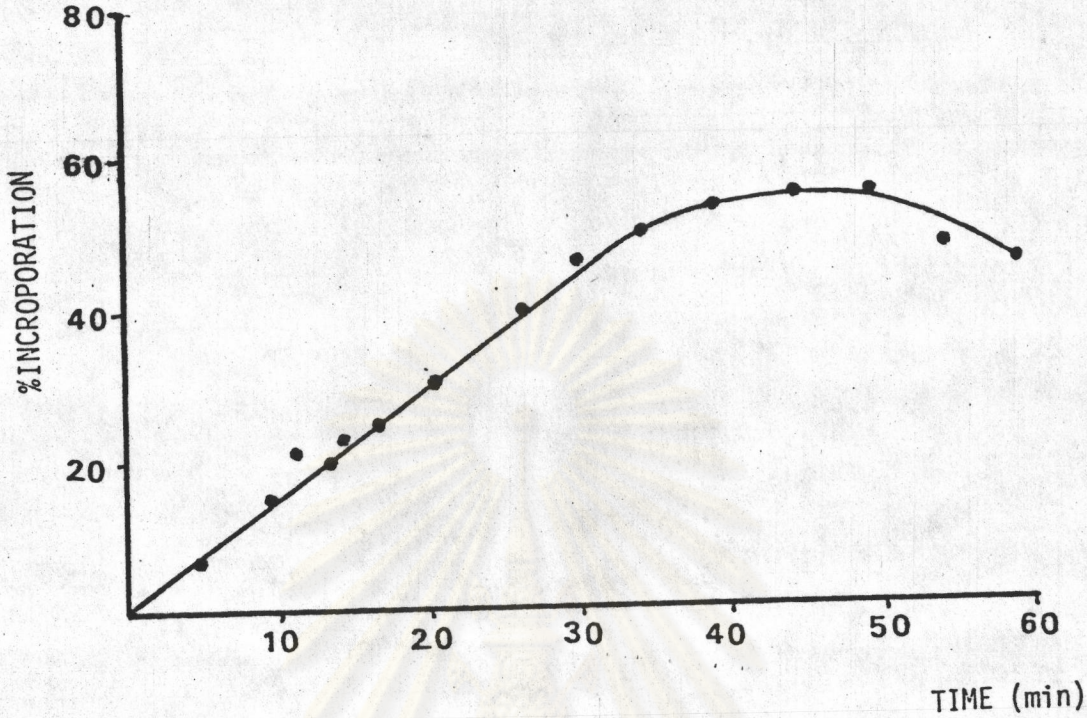


Figure 23. The kinetics of nick translation reaction.

The pSA30 (200 ng) was labelled with 50 μCi (α -^{32}P)-dATP in 50 mM Tris-HCl, pH 7.5 at 15° C. The reaction mixture also contained 10 mM $MgCl_2$, 50 μg/ml BSA, 1mM DTT, 10 uM dNTPs (d CTP, d GTP and d TTP), 1 pg DNase and 3 unit of DNA polymerase I. The % incorporation of (α -^{32}P)-dATP into DNA was plotted verus the incubation time.

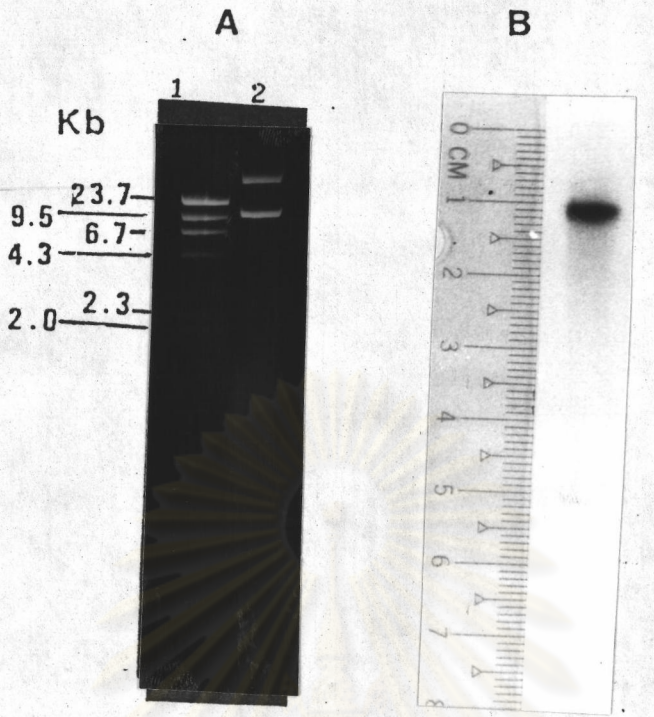


Figure 24. The electrophoretic mobility of ^{32}P-labelled pSA30 and native pSA30 on agarose gel.

The ^{32}P-labelled pSA30 and native pSA30 were subjected to electrophoresis on 1 % agarose gel in Tris-borate buffer pH 8.3, and then the marker gel was stained with ethidium bromide, the photograph of the gel was taken under UV light whereas the agarose gel contain labelled pSA30 was dried and exposed to X-ray film at-70 °C for 2 h.

A) lane 1, size markers of λ /Hind III fragments
lane 2, crude pSA30 plasmid

B) Autoradiograph of ^{32}P-labelled of purified pSA30 on dried agarose gel.

Further, the ability to form DNA-DNA hybrid molecules can be exploited to locate particular genes in a given fragment of nucleic acid. Thus, two techniques of nucleic acid hybridization were used in this studies.

1. Southern blot hybridization

In principle of Southern blot hybridization is that, DNA fragments that have been electrophoretically separated on agarose gel are transfered to a nitrocellulose filter via capillary action prior to hybridization. DNAs are fixed onto nitrocellulose filter in the exact replicas of their gel separations.

1.1 Condition for Southern blot transfer

This experiment was designed to test for appropriate condition of transfer by using sheared calf thymus DNA as a control. Concentration of 1 to 25 ug of sheared calf thymus DNA were electrophoresed on 0.7 % agarose gel (figure 25A). After they were transfered to nitrocellulose filter by Southern blotting method as described in Materials and Methods, the remained DNA on the gel was checked by ethidium bromide staining (figure 25B).

This result indicates that small DNA fragments could be transfered to nitrocellulose filter in higher efficiency than large DNA fragments. Yield of transfer is approximately 50 % or more in this condition.

1.2 Detection of *nif* structural genes on plasmid of *Azospirillum spp*.

In the present study, we attampted to find the answer wheather *nif* structural genes are on plasmid of *Azospirillum*

spp. So, the homology between nif structural gene of Klebsiella pneumoniae on pSA30 and Azospirillum plasmids were studied by Southern blot hybridization experiment.

Plasmids were isolated from three strains of Azospirillum spp. by the method of Hansen and Olsen (1978). It purity is shown in figure 26 A and the autoradiogram of hybridized DNA is shown in figure 26B. The pRD1 plasmid which carry nif operon of Klebsiella pneumoniae (Dixon et al., 1976) was used as positive control as well as a molecular weight size marker. To prevent from over hybridization of positive control, the nitrocellulose filter containing pRD1 was cut and the hybridization was performed in separated bag with ten-fold of labelled probe less than the hybridization of Azospirillum plasmids. From figure 26B, the hybridization could not be observed in the plasmid of Azospirillum whereas one predominant hybridization band was found in pRD1 position (lane 2).

This result suggested that the nif structural genes of Azospirillum spp., might not locate on the plasmid. Therefore, it is likely that the nif structural genes of A.brasilense Sp7, A. lipoferum SpMRAI and Azospirillum spp A12 located on their chromosomes, which are similar to A. brasilense A2 and five strains of Azotobacter spp.

1.3 Detection of nif structural genes on restriction fragments of chromosomal DNA.

This experiment was performed to investigate restriction fragments of various chromosomal DNA from Azotobacter and Azospirillum which showed homology to Kelbsiella pneumoniae nif

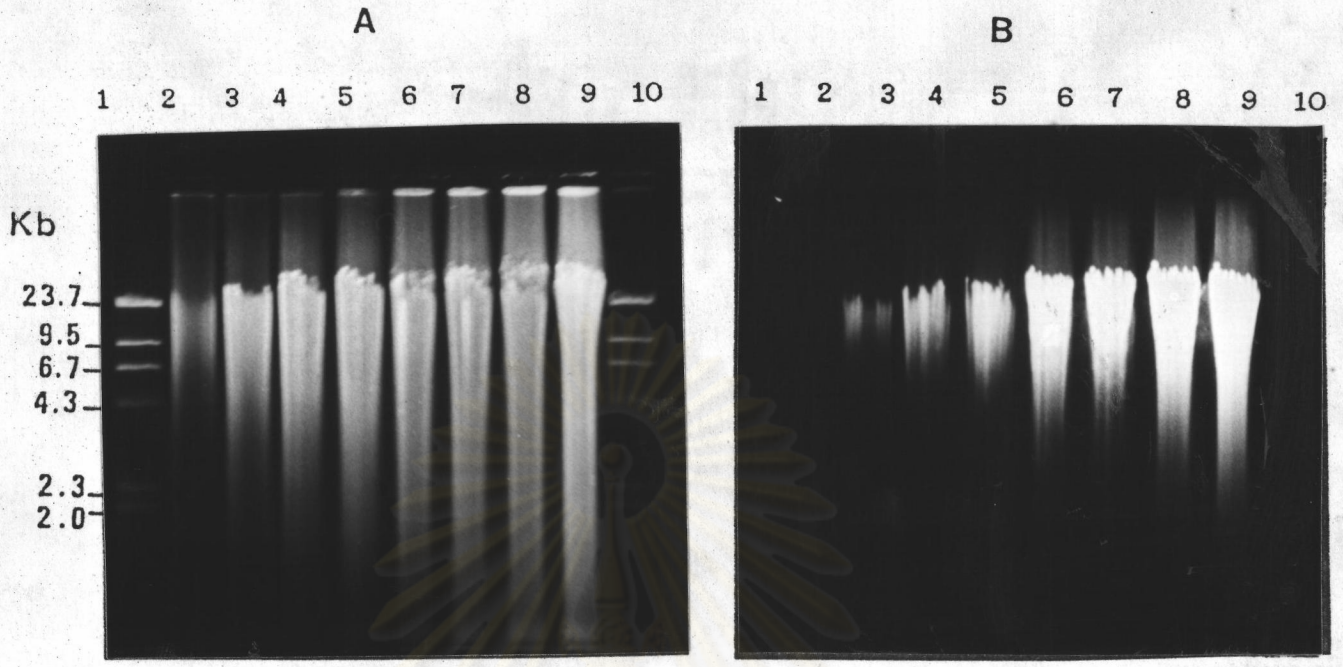


Figure 25. Ethidium bromide staining pattern of sheared calf thymus DNA on agarose gel.

Calf thymus DNA was sheared by pass through a 26Gx1/2" needle for several times and then were load on 0.7 % agarose gel. Electrophoresis was performed in Tris-borate buffer, pH 8.3. After staining with ethidium bromide, the photograph was taken under UV light (A), and then Southern blot was performed for 12 h. The gel was stained with ethidium bromide and photograph was taken under UV light again (B).

Lane (1) and (10) are 1 μg of λ DNA digested with Hind III. Lane (2) to (9) are sheared calf thymus DNA, concentration of 1, 3, 5, 7, 10, 15, 20 and 25 μg, respectively.

Figure 26 A. Ethidium bromide staining pattern of plasmid on agarose gel.

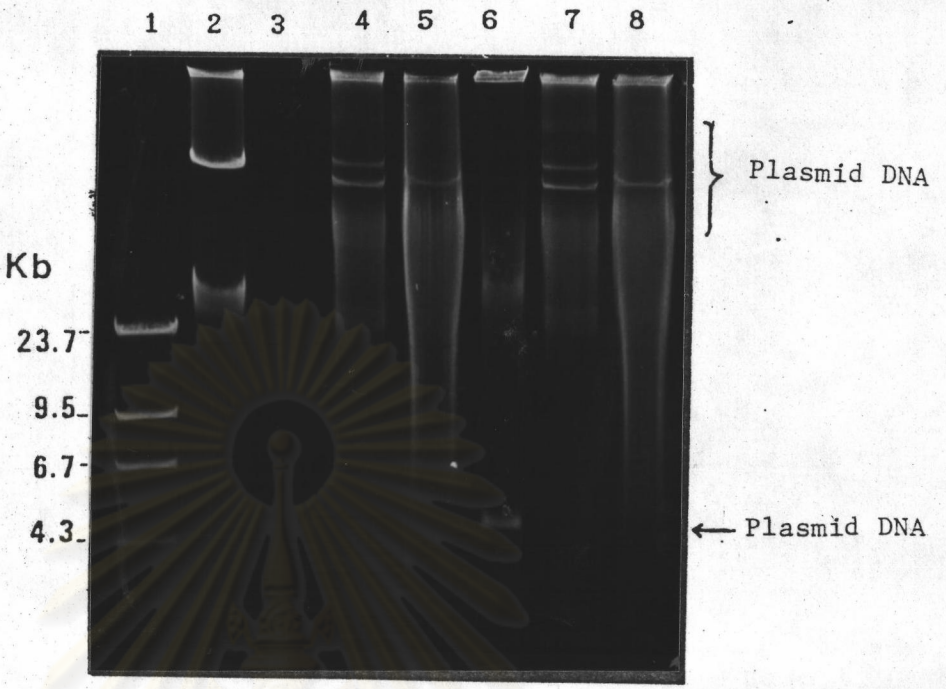
The extracted plasmid of Azospirillum were prepared by the method of Hansen and Olsen. After electrophoresed on 0.7 % agarose gel, the gel was stained with ethidium bromide then photograph was taken under UV light.

Lane 1 is λ /Hind III; lane 2 is pRD1; lane 3 is flank; lane 4 and lane 7 are Azospirillum spp. A12 plasmid; lane 5 and lane 8 are Azospirillum brasilense Sp7 plasmid; lane 6 is Azospirillum lipoferum SpMRAI plasmid.

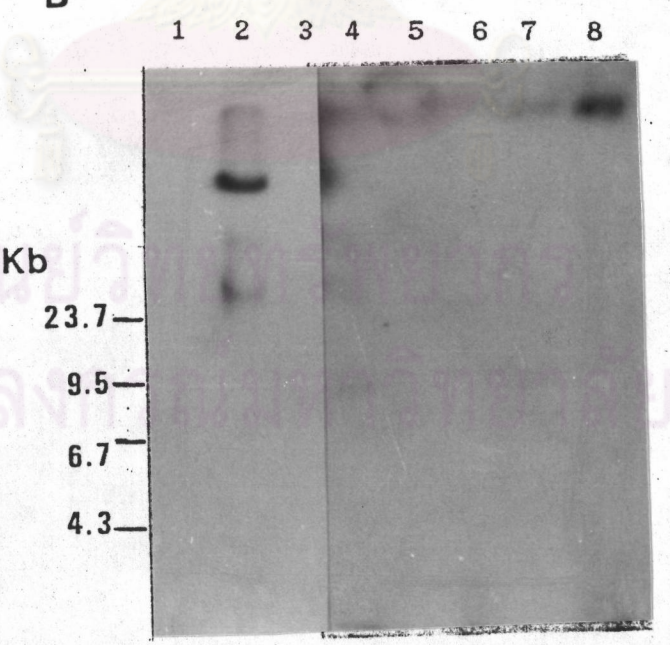
Figure 26 B. Autoradiograph of the nitrocellulose filter containing plasmids of Azospirillum after hybridized with ^{32}P-labelled pSA30.

The gel in figure 26 A was transferred onto nitrocellulose filter and then was hybridized with ^{32}P-labelled pSA30 (specific activity of 3×10^7 cpm/μg DNA and 3×10^6 cpm was added) at 42°C for 48 h. The filter was washed at 50°C then exposed to X-ray film at -70°C for 14 days with intensifying screens.

A


1 2 3 4 5 6 7 8
Plasmid DNA
Kb
23.7
9.5
6.7
4.3
Plasmid DNA

B


1 2 3 4 5 6 7 8
Kb
23.7
9.5
6.7
4.3

structural genes by Southern hybridization techniquees., using the ^{32}P-labelled pSA30 as a probe.

After digestion of chromosomal DNA with various restriction endonuclease, the restriction fragments were size fractionated by electrophoresis on agarose gel and then transferred onto nitrocellulose filters. These filters were hybridized with ^{32}P-labelled pSA30.

Southern hybridization of restriction endonuclease digested Azotobacter and Azospirillum chromosomal DNA were showed in figure 27-32. Linear black bands in the position of gel slot on the autoradiograme are interpreted to hybridization bands, and they were interpreted to contain DNA representing sequences which have homologous to nif structural genes of Klebsiella pneumoniae. The size of hybridization bands were calculated from the mobility curves of standard molecular size marker on each gel were summarized in Table 7. The smallest size of DNA fragments could be presented on the gel is 500 base pair for 0.7 % agarose gel and 300 base pair for 0.8 % agarose gel in this electrophoretic condition. From table 7, for example, the results showed that homology detected between the Klebsiella pneumoniae probe and A. vinelandii KT1 DNA was limited to a 5 kb HindIII fragment, a larger 30 kb BglII fragment and three EcoRI fragments.

The results illustrated the ability of nif structural genes from K. pneumoniae to hybridize with some restriction fragments of chromosomal DNA from all strains studied. Thus, these results also clearly show that the nif structural genes of all Azotobacter and Azospirillum strains locate on their chromosomes. In addition, the hybridization patterns of their nif structural genes showed difference among the species and strains.

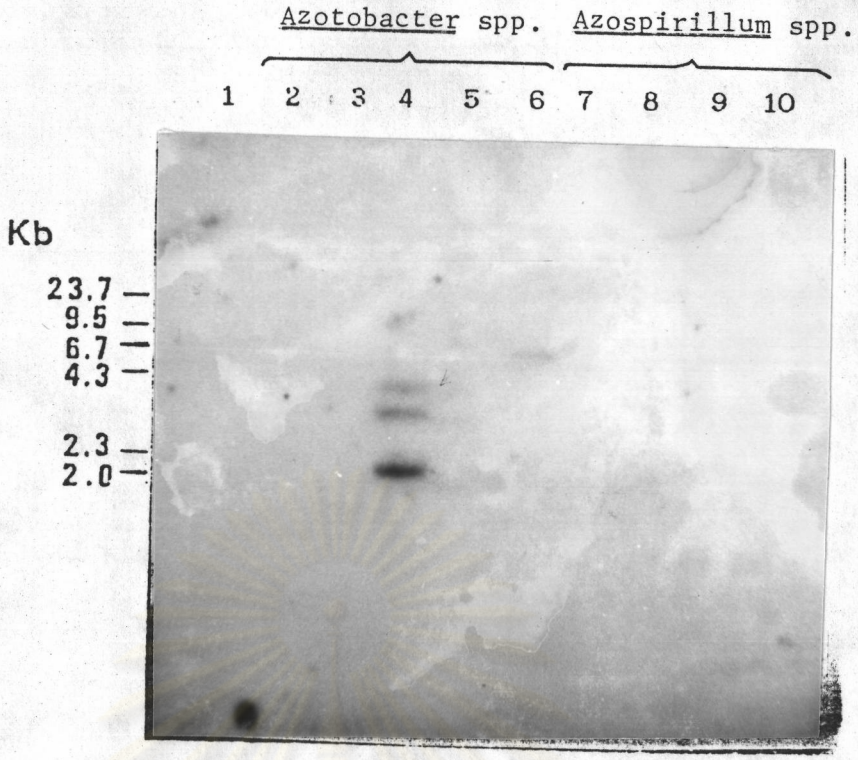


Figure 27. Autoradiograph of nitrocellulose filter containing the Sma I-digested fragments of various species and strains of Azotobacter and Azospirillum after hybridized with ^{32}P-labelled pSA30.

The gel in figure 9 was transferred onto nitrocellulose filter and then was hybridized with ^{32}P-labelled pSA30 (specific activity of 2.3×10^7 cpm/μg DNA and 6×10^6 cpm was added). The hybridization was performed at 42°C for 48 h. The filter was washed at 50°C and exposed to X-ray film at -70°C for 14 days with intensifying screens.

lane 1, λ DNA digested with Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRA1
lane 10, Azospirillum brasilense A2

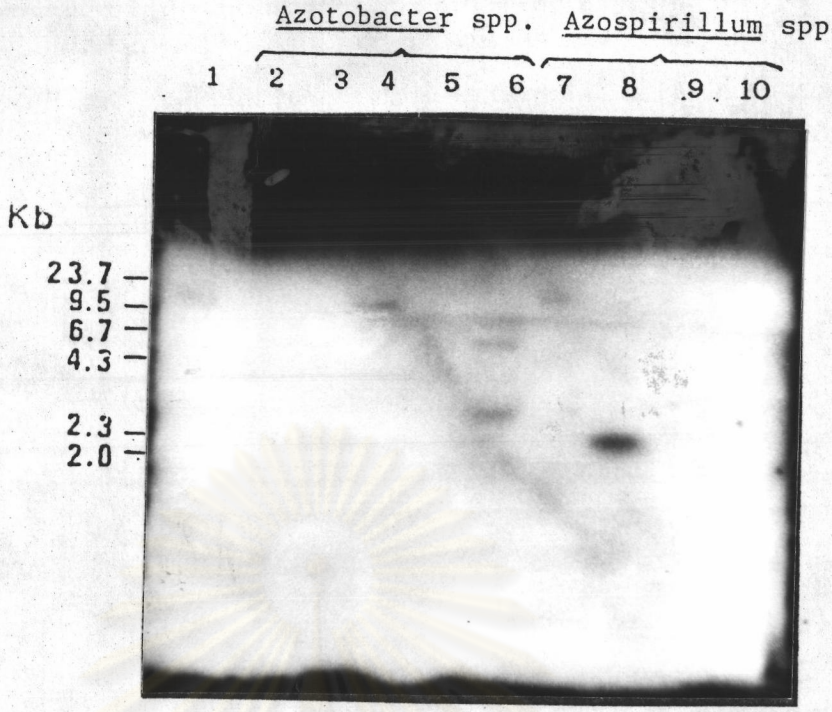


Figure 28. Autoradiograph of nitrocellulose filter containing the Bgl II-digested fragments of various species and strains of Azotobacter and Azospirillum after hybridized with ^{32}P-labelled pSA30.

The gel in figure 10 was transferred onto nitrocellulose filter and then was hybridized with ^{32}P-labelled pSA30 (specific activity of 2.5×10^7 cpm/μg DNA and 6.5×10^6 cpm was added). The hybridization was performed at 42° C for 48 h. The filter was washed at 50° C and exposed to X-ray film at -70° C for 14 days with intensifying screens

lane 1, λ DNA digested with Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azotobacter chroococcum NP
lane 7, Azospirillum brasilense Sp7
lane 8, Azospirillum spp. A12
lane 9, Azospirillum lipoferum SpMRA1
lane 10, Azospirillum brasilense A2

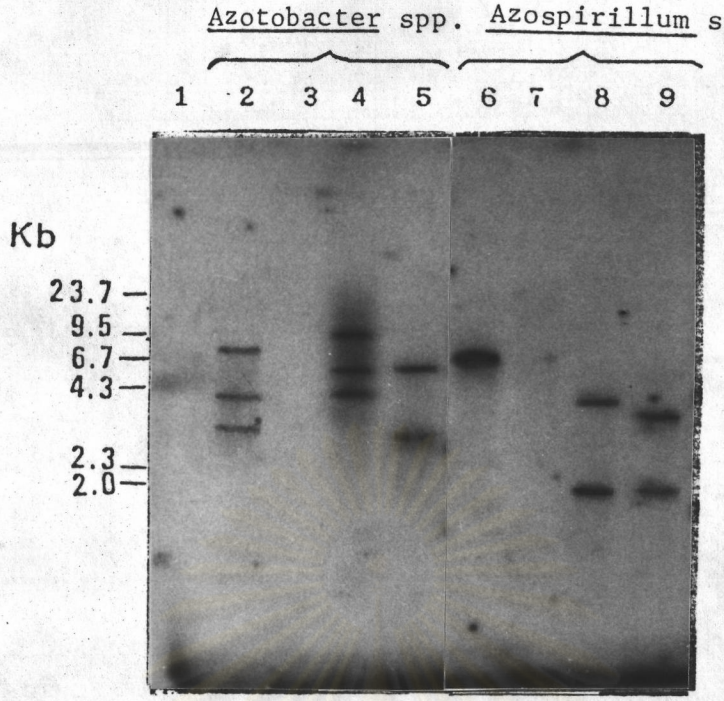


Figure 29. Autoradiograph of the nitrocellulose filter containing the EcoRI-digested fragments of various species and strains of Azotobacter and Azospirillum after hybridized with ^{32}P-labelled pSA30.

The EcoRI-digested fragments of Azotobacter spp. and Azospirillum spp. were fixed onto nitrocellulose filter and then was hybridized with ^{32}P-labelled pSA30 (specific activity of 2.5×10^7 cpm/μg DNA and 6.5×10^6 cpm was added). The hybridization was performed at 42° C for 48 h. The filter was washed at 50° C and exposed to X-ray film at -70° C for 14 days with intensifying screens.

lane 1, λ /Hind III
lane 2, Azotobacter vinelandii KT1
lane 3, Azotobacter vinelandii KT2
lane 4, Azotobacter chroococcum KT
lane 5, Azotobacter paspali B
lane 6, Azospirillum brasilense Sp7
lane 7, Azospirillum spp. A12
lane 8, Azospirillum lipoferum SpMRA1
lane 9, Azospirillum brasilense A2

Figure 30 A. Ethidium bromide staining pattern of restriction fragments of chromosomal DNA from Azotobacter.

Four μg of DNA were digested with 100 units of Bam HI or 35 units of Hind III or 50 units of Sal I, then electrophoresed on 0.8 % agarose gel. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III

lane 2, Azotobacter vinelandii KT1/Hind III

lane 3, Azotobacter vinelandii KT2/Hind III

lane 4, Azotobacter chroococcum KT/Hind III

lane 5, Azotobacter paspali B/Hind III

lane 6, Azotobacter vinelandii KT1/Bam HI

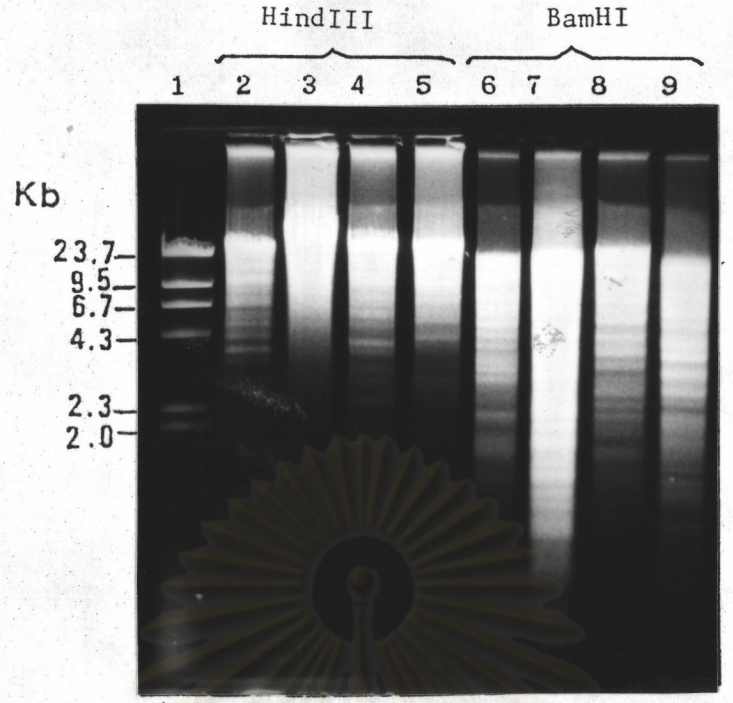
lane 7, Azotobacter vinelandii KT2/Bam HI

lane 8, Azotobacter chroococcum KT/Bam HI

lane 9, Azotobacter paspali B/Bam H

Figure 30.B. Autoradiograph of the nitrocellulose filter containing the restriction fragments of chromosomal DNA after hybridized with ^{32}P-labelled pSA30.

The gel in figure A was transferred onto nitrocellulose filter and then were hybridized with ^{32}P-labelled pSA30 (specific activity 2.5×10^7 cpm/μg DNA and 6.5×10^6 cpm was added). The hybridization was performed at 42°C for 48 h. The filter was washed at 50°C and exposed to X-ray film at -70°C for 14 days with intensifying screens.


HindIII
BamHI
A
1 2 3 4 5 6 7 8 9
Kb
23.7
9.5
6.7
4.3
2.3
2.0

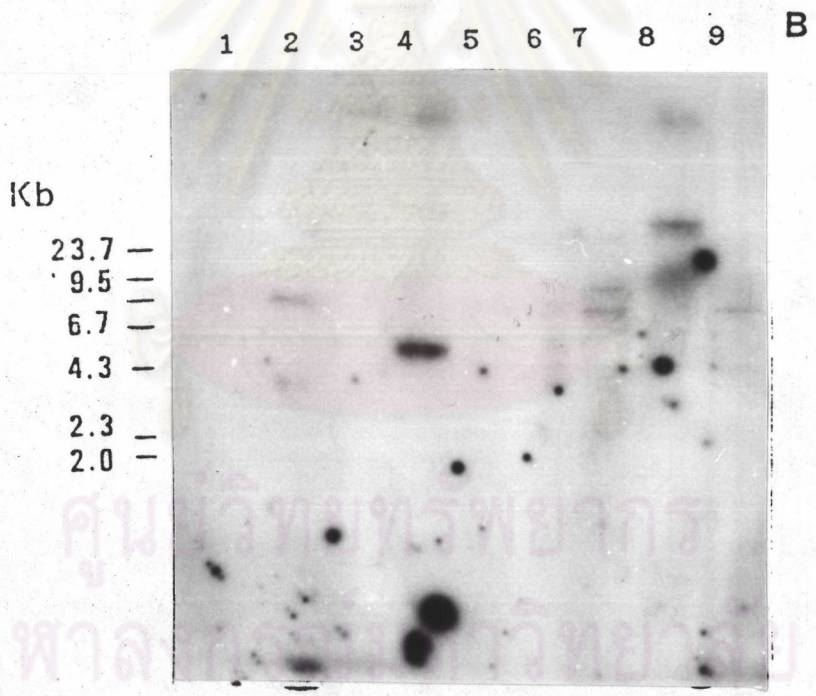

B
1 2 3 4 5 6 7 8 9
Kb
23.7
9.5
6.7
4.3
2.3
2.0

Figure 31 A. Ethidium bromide staining pattern of restriction fragments of chromosomal DNA from Azospirillum.

Four μg of DNA were digested with 100 units of Bam HI or 350 units of Hind III then electrophoresed on 0.8 % agarose gel. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III

lane 2, Azospirillum brasilense Sp7/Bam HI

lane 3, Azospirillum spp. A12/Bam HI

lane 4, Azospirillum lipoferum SpMRA1/Bam HI

lane 5, Azospirillum brasilense A2/Bam HI

lane 6, Azospirillum brasilense Sp7/Hind III

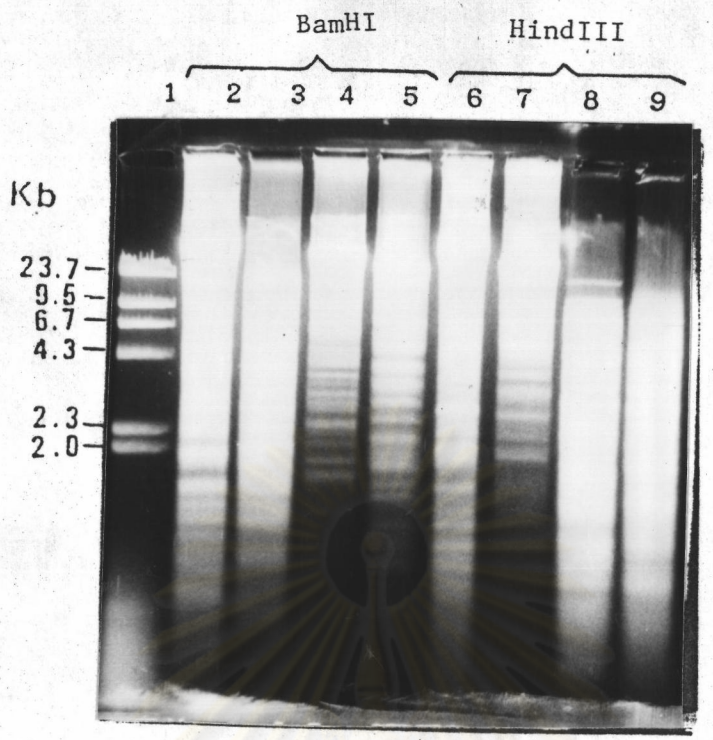
lane 7, Azospirillum spp. A12/Hind III

lane 8, Azospirillum lipoferum SpMRA1/Hind III

lane 9, Azospirillum brasilense A2/Hind III

Figure 31 B. Autoradiograph of the nitrocellulose filter containing the restriction fragments of chromosomal DNA after hybridized with ^{32}P-labelled pSA30.

The gel in figure 31 A was transferred onto nitrocellulose filter and then were hybridized with ^{32}P-labelled pSA30 (specific activity of 2.5×10^7 cpm/μg DNA and 6.5×10^6 cpm was added). The hybridization was performed at 42 °C for 48 h. The filter was washed at 50 °C and exposed to X-ray film at -70 °C for 14 days with intensifying screens.


BamHI
HindIII
A
1 2 3 4 5 6 7 8 9
Kb
23.7
9.5
6.7
4.3
2.3
2.0

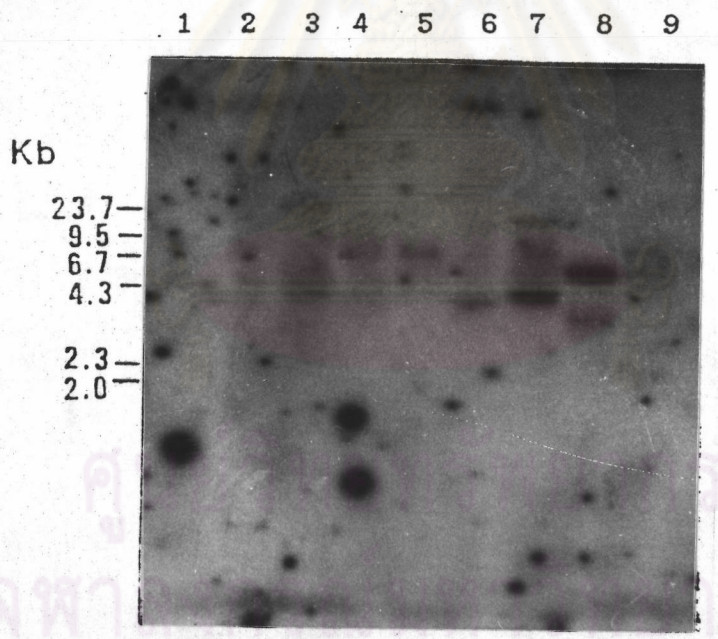

1 2 3 4 5 6 7 8 9
B
Kb
23.7
9.5
6.7
4.3
2.3
2.0

Figure 32 A. Ethidium bromide staining pattern of Pst I digested fragments of chromosomal DNA from Azospirillum.

Four µg of DNA were digested with 80 units of Pst I were electrophoresed on 0.8 % agarose gel. After staining with ethidium bromide, photograph was taken under UV light.

lane 1, λ /Hind III

lane 2, —

lane 3, Azospirillum lipoferum SpMRA1

lane 4, Azospirillum brasilense A2

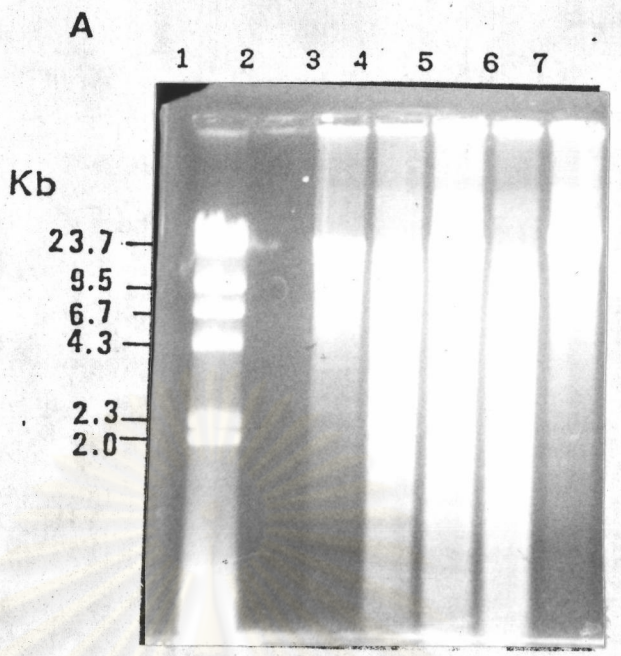
lane 5, Azospirillum brasilense Sp7

lane 6, Azospirillum spp. A12

lane 7, Azospirillum spp. A12/EcoRI

Figure 32 B. Autoradiograph of the nitrocellulose filter containing the restriction fragments of chromosomal DNA after hybridized with ^{32}P-labelled pSA30.

The gel in figure 32 A was transferred onto nitrocellulose filter and then were hybridized with ^{32}P-labelled pSA30 (specific activity of 2.5×10^7 cpm/ug DNA and 6.5×10^6 cpm was added). The hybridization was performed at 42°C for 48 H. The filter was washed at 50°C and exposed to X-ray film at -70°C for 14 days with intensifying screens.



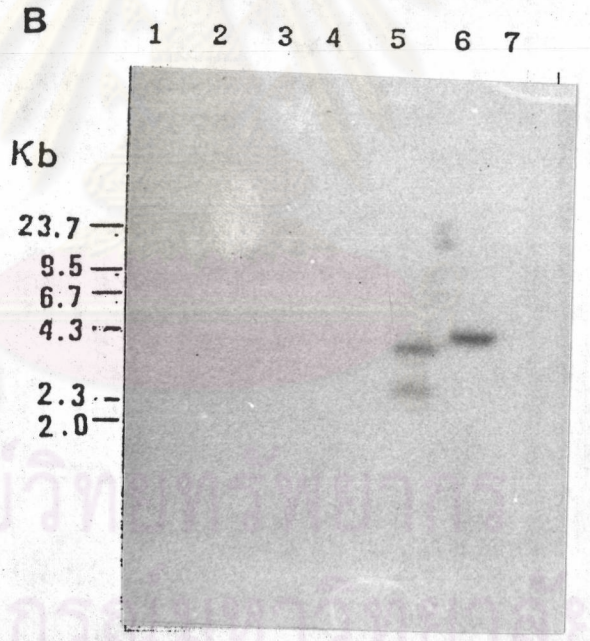


Table 7. Restriction analysis of *nif* structural genes of *Azotobacer* spp. and *Azospirillum* spp. by Southern hybridization when use *nif* structural genes of *Klebsiella pneumoniae* on pSA30 as a hybridization probe.

Bacterial strains	Restriction hybridization fragment (kb)					
	Bam HI	EcoRI	Hind III	Bgl II	SmaI	Pst I
I *Azotobacter* spp.						
A.vinelandii KT1	-	8.7,4,3	5	30	-	ND
A.vinelandii KT2	5.2,3,8	-	-	30	-	ND
A.chroococcum KT	30,8.2	9.7,6,4.2	3.3	30,13	3.9,3.2,2.1	ND
A.chroococcum NP	ND	ND	ND	6,2.9	5.9	ND
A.paspali B	5	6.1,3	-	30	-	ND
II *Azospirillum* spp.						
A.brasilense Sp7	-	6.7	3.8	17	-	3.9,2.7
A.brasilense A2	6.9	3.4,1.6	7.8,5.6	-	-	-
A.lipoferum SpMRA1	6.2	4,1.9	5.1,3.1	-	-	-
Azospirillum spp.A12	-	-	15,3.8	2.4	-	4.3

ND = not determined

- = negative result

2. Dot blot hybridization of Azotobacter and Azospirillum chromosomal DNA and pSA30 with labelled pACYC184

In order to assure that hybridization in this study was the property of nif structural genes inserted pSA30. Thus, the homology between the vector DNA (pACYC184) and variety of DNA were tested by dot blot hybridization. The denatured chromosomal DNA of Azotobacter and Azospirillum were fixed onto nitrocellulose filter then was prehybridized and hybridized with labelled pACYC184. After the filter was washed to remove the non-hybridized probe. The hybrid formed was detected autoradiography. Calf thymus DNA and pSA30 plasmid were used as negative and positive control respectively. The photographic picture of autoradiogram is shown in figure 33.

Determination of nif structural genes fragments homology by dot blot hybridization

In an attempt to determine the homology between various fragments of nif structural genes from Klebsiella pneumoniae which carry on pSA30 (figure 3) and nif structural genes on chromosomal DNA of Azotobacter or Azospirillum, dot blot hybridization technique has been performed.

Two hundred and fifty nanograms and 500 nanograms of denatured chromosomal DNA from five strains of Azotobacter and four strains of Azospirillum were bound to nitrocellulose filters and hybridized with ^{32}P-labelled of nif fragment A1 (part of nif K) or nif fragment A2 (part of nif D) or nif fragment A3 (nif H and part of nif D). One sheet of nitrocellulose filter which carry chromosomal DNA from five strains of Azotobacter and the other sheet carry chromosomal DNA from four strains of Azospirillum were hybridized in the same bag with

each labelled probe.

Twenty five nanograms of DNA from Klebsiella pneumoniae and 500 ng of calf thymus DNA were used as positive and negative control in these experiments, respectively.

After washing, the filters were autoradiographed and the autoradiograms are shown in figure 34. Then the nitrocellulose filters were cut into even pieces of 1x1 in^2. The piece of nitrocellulose was immersed in a vial containing 5 ml of Bray's solution and counted for radioactivity. The backgrounw radioactivity was obtained from counting the nitrocellulose filters without DNA which traeted exactly the same condition.

Dot blot hybridization kinetic curves and the autoradiograms when used nif fragment A1, nif fragment A2 and nif fragment A3 as a hybridization probes are shown in figure 35, 36 and 37, respectively. The results showed that when increasing amount of chromosomal DNA were hybridized with a constant amount of ^{32}P-labelled probes, the linear relationship at first order of the kinetic curves and some maximum hybridization were obtained. These indicated that the homology could be determine by comparing Δcpm/ Δ ng DNA at the first order to Δcpm/Δng DNA which obtained from hybridization curve of Klebsiella pneumoniae chromosome. Thus the homology was calculated and summarized in Table 8.

Table 8 shows that the degree of homology between nif K of Klebsiella pneumoniae (nif fragment A1) and four strains of Azospirillum chromosomal DNA was very low (1.7-2.0 %). This evidence is not observed from Azotobacter chromosomal DNA since the homology of nif K in these organism was in the range of 5.0-9.6 %. By using

the nif fragment A2 (part of nif D) of Klebsiella pneumoniae as a hybridization probe, high degree of homology of nif D was observed in Azotobacter vinelandii KT2 (15.9%), Azotobacter chroococcum KT (13.3 %) and Azospirillum brasilense Sp7 (14.4 %) whereas low degree of homology was obtained from Azotobacter vinelandii KTI (3.4 %) and Azospirillum spp. A12 (3 %). The high degree of homology between nif fragment A3 carrying nif H and part of nif D of Klebsiella penumoniae and Azotobacter or Azospirillum chromosomal DNA was observed from Azotobacter vinelandii KT2 (11.9 %) and Azospirillum brasilense Sp7 (8 %). The low degree of homology was seen in Azotobacter vinelandii KTl (3.4 %) and Azospirillum lipoferum SpMRAl (3.1 %).

Furthermore, the homology among the labelled probes (nif fragment Al, A2 and A3) were determined in term of ug of hybridized probe/kb of genome. Two factors; the specific activity and size of the labelled probes are used for calculation (see appendix). The result was summarized in table 9. The result showed that the nif fragment A2 which contains the region of nif D of Klebsiella pneumoniae hybridized to the chromosomal DNA from three strains of Azotobacter spp. (KT2, KT and NP) in higher degree of homology (7.9-20.7x10^{-8} µg of probe/kb of genome) than the nif fragment A3 which carrying nif H and part of nif D (rangeing from 5.2-14.6x10^{-8} µg of probe/kb of genome). The nif fragment Al which carrying nif K gave the lowest degree of homology (4.5-8.3x10^{-8}µg of probe/kb of genome). In Azotobacter paspaliB, the highest degree of homology (8.5x10^{-8} µg of probe/kb of genome) obtained from the hybridization when the nif fragment Al (nif K) was used as probe. Whereas difference of degree of homology among the three fragments of nif structural genes is not

seen in Azotobacter vinelandii KT1. All strains of Azospirillum spp. showed that very low degree of homology (ranging from 1.5 - 1.8×10^{-8} µg of probe/kb of genome) was found in the region of nif K (nif fragment A1) whereas the high degree of homology (ranging from 3.9 - 18.7×10^{-8} µg of probe/kb of genome) was seen in the region of nif D (nif fragment A2)

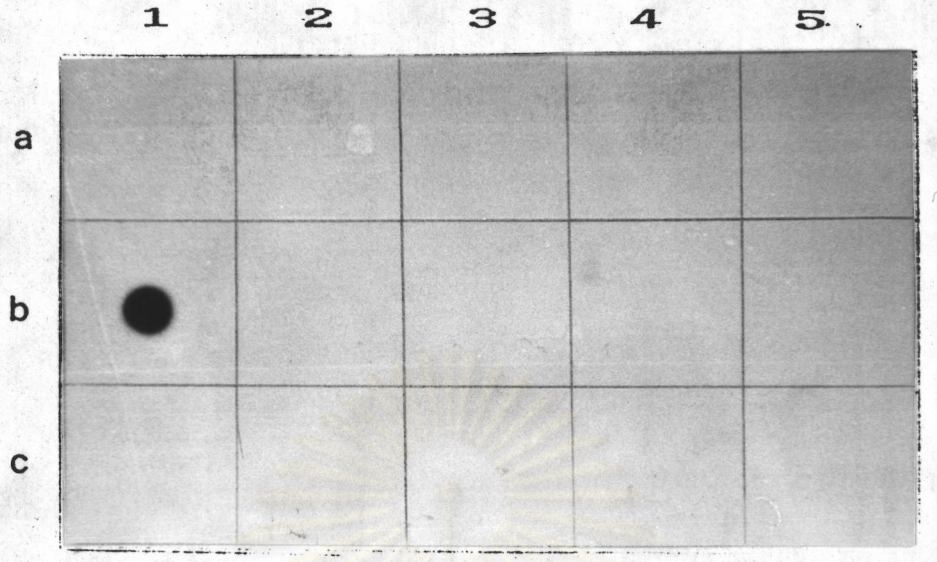


Figure 33. Autoradiograph of dot blot filter containing chromosomal DNA of Azotobacter, Azospirillum and pSA30 after hybridized with ^{32}P-labelled pACYC184 fragment.

The filter was hybridized with ^{32}P-labelled pACYC184 fragment (specific activity of 4.2×10^7 cpm/ug DNA was added). Hybridization was performed at 42°C for 48 h and washed at 50°C. Then the filter was exposed to X-ray film at -70°C for 24 h with intensifying screens.

(a, 1) 500 ng of K.pneumoniae M5a1 DNA
(a, 2) 500 ng of calf thymus DNA
(a, 3) -
(a, 4) -
(a, 5) -
(b, 1) 2 ng of pSA30 plasmid
(b, 2) 500 ng of A. brasilense Sp7-DNA
(b, 3) 500 ng of Azospirillum spp. A12-DNA
(b, 4) 500 ng of A. lipoferum SpMRAI-DNA
(b, 5) 500 ng of A. brasilense A2-DNA
(c, 1) 500 ng of A. vinelandii KT1
(c, 2) 500 ng of A. vinelandii KT2
(c, 3) 500 ng of A. chroococcum KT
(c, 4) 500 ng of A. paspali B
(c, 5) 500 ng of A. chroococcum NP

Figure 34. Autoradiographs of dot blot filter containing chromosomal DNA of Azotobacter (X) and Azospirillum (Y) after hybridized with ^{32}P-labelled nif fragment A1, nif fragment A2 and nif fragment A3.

The filter X and filter Y were hybridized in the same bag with ^{32}P-labelled nif fragment A1 (specific activity = 2.9×10^7 cpm/ug DNA), nif fragment A2 (specific activity = 1.5×10^8 cpm /ug DNA) and nif fragment A3 (specific activity = 3.6×10^7 cpm /ug DNA). Hybridization were performed at 42°C for 48 h and washed at 50°C. Then the filters were exposed to X-ray film at -70°C for 48 h with intensifying screens.

(X) (a,1), 25 ng of K.pneumoniae M5a1 DNA
(a,2), 500 ng of calf thymus DNA
(a,3), blank
(a,4), and (a,5) -
(b,1), 250 ng of A.vinelandii KT1-DNA
(b,2), 250 ng of A.vinelandii KT2-DNA
(b,3), 250 ng of A.chroococcum KT-DNA
(b,4), 250 ng of A.paspali B-DNA
(b,5), 250 ng of A.chroococcum NP-DNA
(c,1), 500 ng of A.vinelandii KT1-DNA
(c,2), 500 ng of A.vinelandii KT2-DNA
(c,3), 500 ng of A.chroococcum KT-DNA
(c,4), 500 ng of A.paspali B-DNA
(c,5), 500 ng of A.chroococcum NP-DNA

(Y) (a,1) 25 ng of K.pneumoniae M5a1
(a,2) 500 ng of calf thymus DNA
(a,3) blank
(a,4) -
(b,1) 250 ng of A.brasilense Sp7-DNA
(b,2) 250 ng of Azospirillum spp. A12-DNA
(b,3) 250 ng of A.lipoferum SpMRAI
(b,4) 250 ng of A.brasilense A2-DNA
(c,1) 500 ng of A.brasilense Sp7-DNA
(c,2) 500 ng of Azospirillum spp.A12
(c,3) 500 ng of A.lipoferum SpMRAI
(c,4) 500 ng of A.brasilense A2

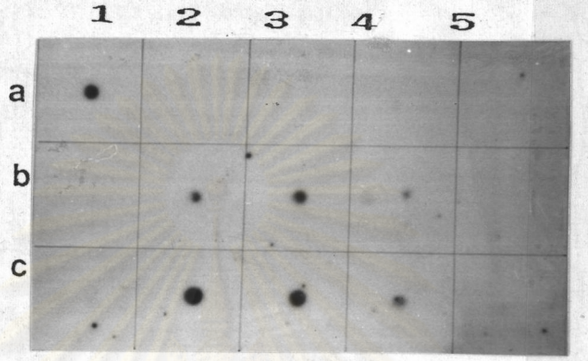
Figure 34.

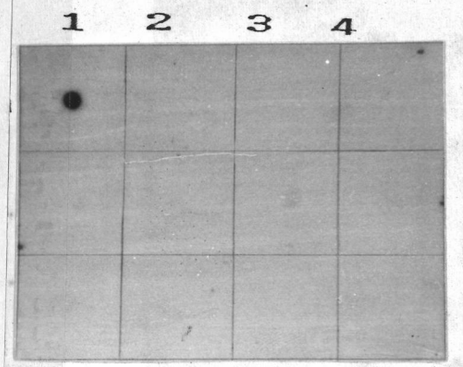
Labelled probes

(X)
Azotobacter

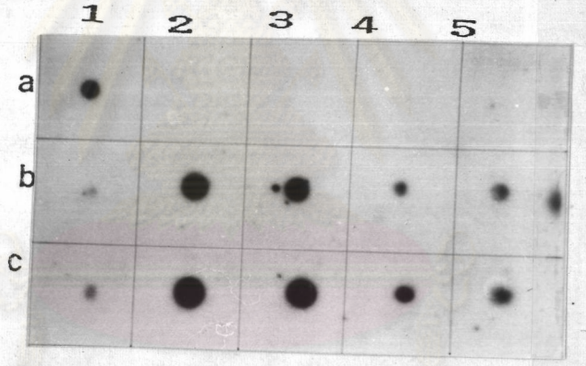
(Y)
Azospirillum

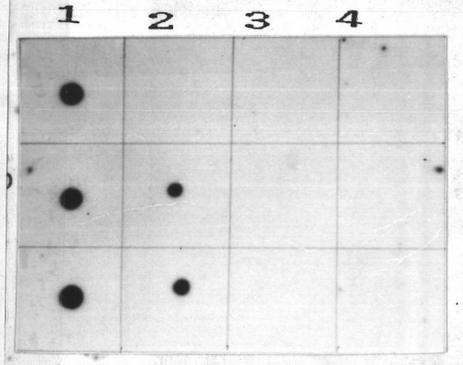
nif fragment A1
(part of nif K)



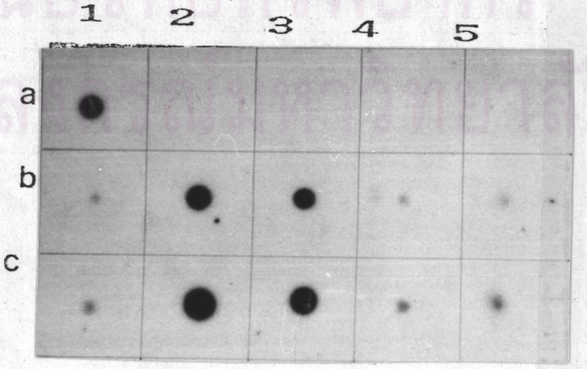


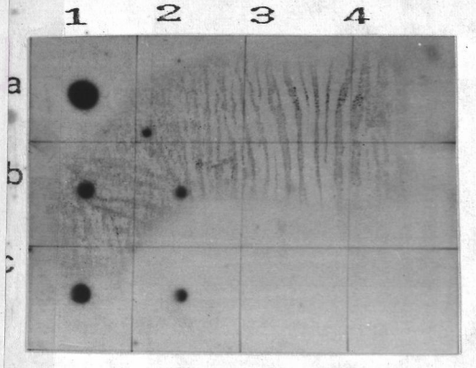
nif fragment A2
(part of nif D)





nif fragment A3
(nif H and part of nif D)





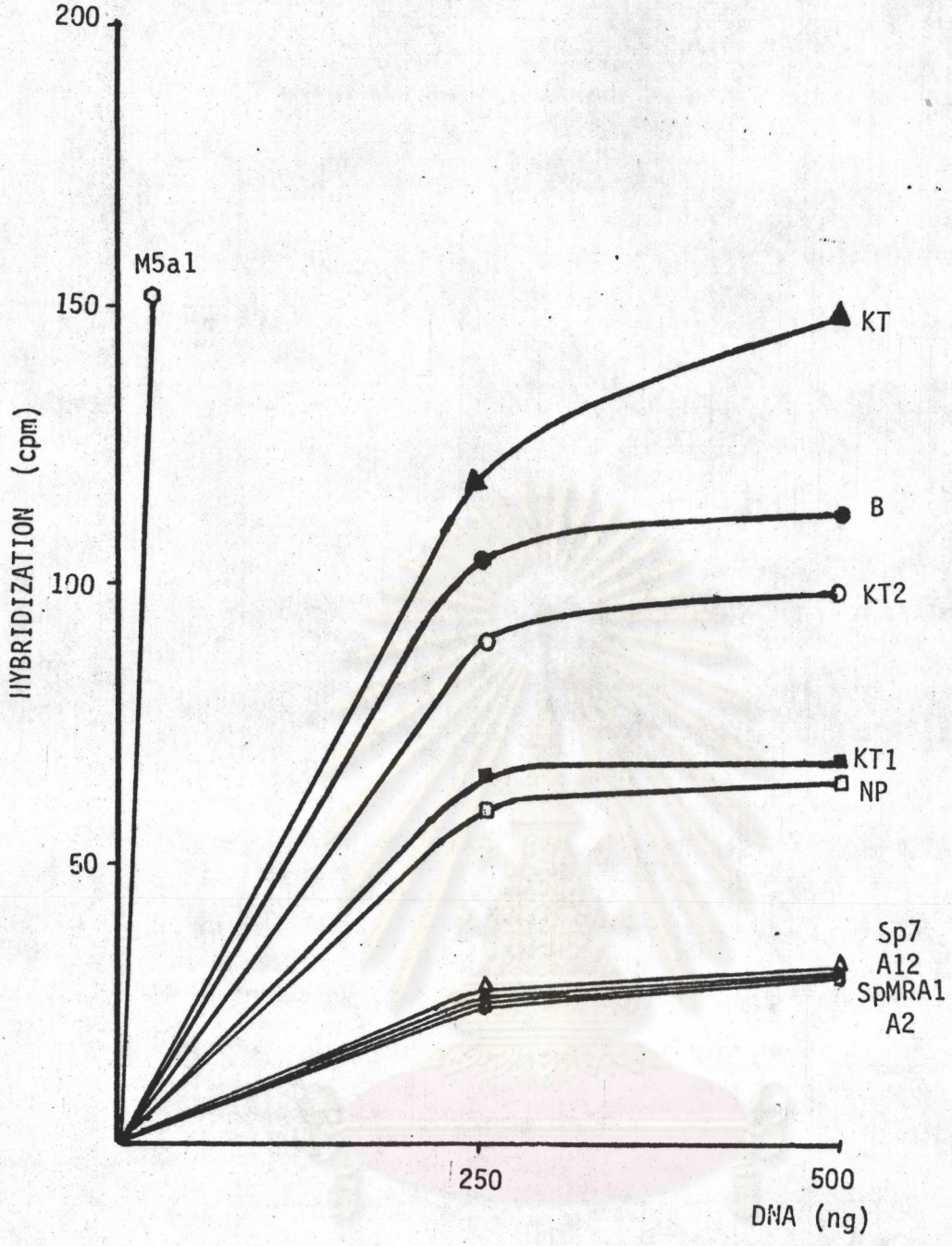


Figure 35. Kinetics of dot blot hybridization of chromosomal DNA of Azotobacter spp. or Azospirillum spp. with ^{32}P-labelled nif fragment A1 (part of nif K).

Variable amounts of chromosomal DNA of Azotobacter and Azospirillum were fixed onto nitrocellulose filters and then hybridized in the same bag with ^{32}P-labelled nif fragment A1 (specific activity = 2.9×10^7 cpm/μg DNA).

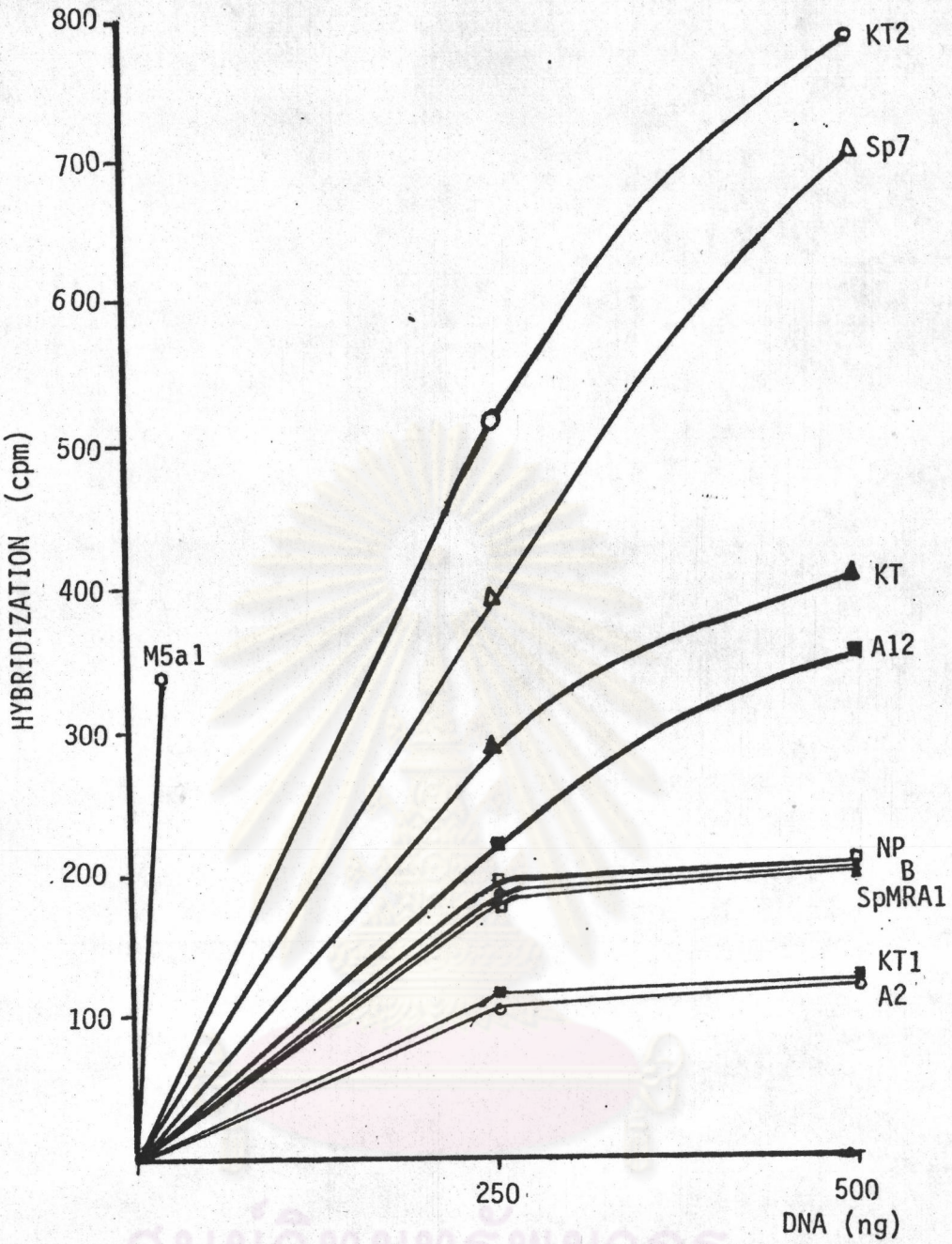


Figure 36. Kinetics of dot blot hybridization of chromosomal DNA of Azotobacter spp. or Azospirillum spp. with ^{32}P-labelled nif fragment A2 (part of nif D).

Variable amounts of chromosomal DNA of Azotobacter and Azospirillum were fixed onto nitrocellulose filters and then hybridized in the same bag with ^{32}P-labelled nif fragment A2 (specific activity = 1.5×10^8 cpm/µg DNA).

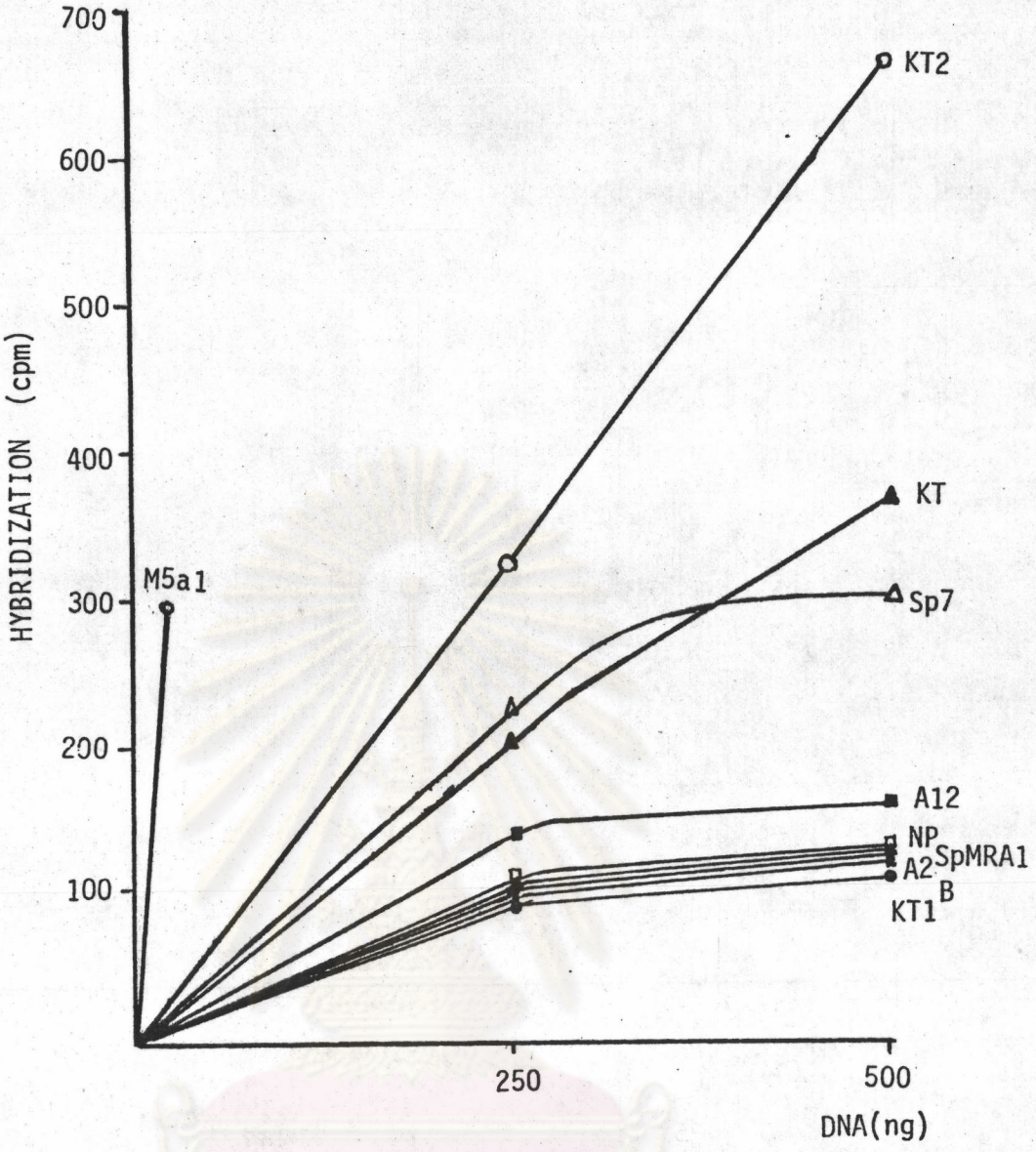


Figure 37. Kinetics of dot blot hybridization of chromosomal DNA of Azotobacter spp. or Azospirillum spp. with ^{32}P-labelled nif fragment A3 (nif H and part of nif D).

Variable amounts of chromosomal DNA of Azotobacter and Azospirillum were fixed onto nitrocellulose filters and then hybridized in the same bag with ^{32}P-labelled nif fragment A3 (specific activity = 3.6×10^7 cpm/μg DNA).

Table 8. The relative homology of nif structural genes fragments between Klebsiella pneumoniae and Azotobacter spp. or Azospirillum spp.

The denatured chromosomal DNA from Azotobacter and Azospirillum were bound to nitrocellulose filter by dot blot method and hybridized with ^{32}P-labelled of nif fragment A1, A2 and A3. After washing, the nitrocellulose filters were cut into even pieces and counted for radioactivity in Bray's solution. The homology were determined by comparing Δcpm/Δng DNA value at the first order of hybridization kinetic curve to Δcpm/Δng DNA value which obtained from hybridization kinetic curve of Klebsiella pneumoniae chromosomal DNA.

Bacterial strains	nif fragment A1 (part of nif K)		nif fragment A2 (part of nif D)		nif fragment A3 (nif Hand part of nif D)	
	Δcpm/Δng DNA	%homology	Δcpm/Δng DNA	%homology	Δcpm/Δng DNA	%homology
Klebsiella pneumoniae M5a1	5.84	100	13.2	100	11.2	100
A. vinelandii KT1	0.36	5.0	0.45	3.4	0.38	3.4
A. vinelandii KT2	0.44	6.2	2.1	15.9	1.33	11.9
A. chroococcum KT	0.58	9.3	1.75	13.3	0.82	7.3
A. chroococcum NP	0.3	5.1	0.8	6.1	0.47	4.2
A. paspali B	0.54	9.6	0.75	5.7	0.41	3.7
A. brasilense Sp7	0.12	2.0	1.9	14.4	0.9	8.0
A. brasilense A2	0.1	1.7	0.9	6.8	0.6	5.4
A. lipoferum SpMRA1	0.1	1.7	0.8	6.1	0.35	3.1
Azospirillum spp. A12	0.1	1.7	0.4	3.0	0.45	4.0

Table 9. The relative homology of *nif* structural genes fragments between *Klebsiella pneumoniae* and *Azotobacter* spp. or *Azospirillum spp.* (inx10^{-8} ug of probe per kb of genome).

Bacterial strains	*nif* fragment A1 (part of *nif* K)	*nif* fragment A2 (part of *nif* D)	*nif* fragment A3 (*nif* Hand part of *nif* D)
Klebsiella pneumoniae M5al	100	100	100
Azotobacter vinelandii KT1	4.4	4.4	4.2
Azotobacter vinelandii KT2	5.5	20.7	14.6
Azotobacter chroococcum KT	8.3	17.3	9.0
Azotobacter chroococcum NP	4.5	7.9	5.2
Azotobacter paspali B	8.5	7.4	4.5
Azotobacter brasilense Sp7	1.8	18.7	9.8
Axotobacter brasilense A2	1.5	8.8	6.6
Azotobacter lipoferm SpMRA1	1.5	7.9	3.8
Azospirillum spp. A12	1.5	3.9	4.9