Arch. Pharm. Res. Vol. 18, No. 1 pp. 31-37, 1995

Instability of Pneumococcus Library in pHC79 and pACYC184

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(Received December 1, 1994)

S. pneumoniae (pneumococcus) gene cloning and library construction in *E. coli* multicopy plasmid has been hampered, in part, by instability problems. In this study, stability of pneumococcus gene library in cosmid vector and pACYC184 was examined. Pneumococcus library in the cosmid vector pHC79 was extremely unstable that most of the recombinant clones were degenerated rapidly. Only 2 out of 849 clones were stable and had appropriate insert size. Pneumococcus library in pACYC184 was also so unstable that the pneumococcal insert and/or part of the vector were deleted. However, the instability problems seemed to be resolved when transcription terminator plasmid was employed for pneumococcus library construction.

Key words: *Streptococcus pneumoniae* library, Recombinant instability

INTRODUCTION

Streptococcus pneumoniae (pneumococcus), a Gram positive nonspore forming coccus, is the causative agent of pneumonia, otitis media, meningitis, and septicemia. In spite of modern antimicrobial agents, it is a leading cause of morbidity and mortality in persons of all ages (Joklik *et al.*, 1988; Storch, 1989). Pneumococcus has also a special feature for genetic transformation, competence (Tomasz, 1966; Morrison and Baker, 1979; Morrison *et al.*, 1982; Vijayakumar and Morrison, 1983), by which it can acquire a variety of genetic information necessary for adaptation and survival. Competence is characterized by the appearance of capacity to process large amounts of DNA for genetic recombination. At competence, double-stranded DNA is bound at the cell surface (Lacks and Greenberg, 1976; Morrison and Guild, 1973), single-stranded fragments are taken into the cell with concomitant degradation of the complementary strand (Lacks, 1962; Lacks and Greenberg, 1973, 1976; Morrison and Guild, 1973a,b), and these fragments are inserted into the homologous chromosome by recombination (Morrison *et al.*, 1983; Rhee and Morison, 1988; Radnis *et al.*, 1990).

It has been known that cloning and characterization of pneumococcus gene has been hampered by the instability of pneumococcus library or instability of recombinants in *E. coli* multicopy plasmid (Chen and Morrison, 1987; Rhee and Morrison, 1988; Chandler and Morrison, 1987). Some pneumococcus genes were successfully cloned using the *E. coli* multicopy plasmid (Garcia *et al.*, 1985, 1986; Paton *et al.*, 1986; Lopez *et al.*, 1987) but others experienced instability of recombinant plasmid or they could not clone pneumococcus genes. In one study, most pneumococcus recombinants in *E. coli* were so unstable that pneumococcal insert and cloning site were deleted (Chen and Morrison, 1987). Cloning of pneumococcus *com* locus in *E. coli* vectors pMB9 and pBR325 failed (Chandler and Morrison, 1987) and attempts to clone the pneumococcus *malM* gene in pBR322 plasmid were unsuccessful (Stassi and Lacks, 1982). And the pneumococcus gene library in *E. coli* vector pUC19 and pJDC10 was unstable: When pUC19 and pJDC10 (both of them originated from replicon of pMB1) were used for shotgun cloning of 9 kb and 12-20 kb of pneumococcus DNA, most of the recombinants were unstable but they might be stabilized by using transcription terminator vectors (Chen and Morrison, 1987). But so far, stability of pneumococcus gene library in *E. coli* vector other than pUC19 and pJDC10 has not been determined. And most of the studies have dealt with cloning problems of individual genes not the problem of pneumococcus library construction. In this study, an *E. coli* plasmid containing p15A replicon, pACYC184, and a cosmid vector pHC79, which can accomodate DNA fragments of upto 40 kb by using headful packaging mechanism of lambda phage, were used for determination of stability of pneumococcus library.

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MATERIALS AND METHODS

Strains and Plasmids

Bacterial strains and plasmids used in this study were described in Table 1.

Reagents

Chemical reagents were purchased from Sigma Chemical Co.(St. Louis, MO) unless otherwise mentioned. 5-dibromo-4-chlo-3-indolygalactoside (X-Gal) and N'-dimethylformamide were from International Biotechnologies Inc. (New Heaven, CT) and Eastman Kodak (Rochester, NY), respectively. Lambda packaging extract was obtained from Amersham Co. (Arlington Heights, IL). Restriction endonucleases, T4 ligase, and alkaline phosphatase were purchased from New England Biolabs (Beverly, MA). Bacterial culture media was purchased from Difco Laboratories (Detroit, MI).

Cultures and media

Pneumococcus culture was grown at 37℃ in CAT broth as described previously (Kim *et al.*, 1992). *E. coli* culture was grown at 37℃ in LB broth (Sambrook *et al.*, 1989). For *E. coli* selection, when required, 20 g of chloramphenicol(Cm), 50 μg of ampicillin(Ap), 15 μg of tetracycline(Tc), or 1 mg of erythromycin(Em) per ml were supplemented.

Recombinant DNA techniques.

Pneumococcal chromosomal DNA was isolated by the technique described previously (Kim *et al.*, 1992). Small scale plasmid preparation for the analysis of recombinant plasmids in transformants were made by alkaline sodium dodecyl sulfate lysis of cultures of *E. coli* grown in the presence of the appropriate antibiotics, as described by Birnboim and Doly (1979)

For large scale preparations, the plasmid DNA was prepared by a modified method of Marko *et al.* (1982) and purified by a CsCl gradient method (Sambrook *et al.*, 1989). Restriction endonucleases, T4 ligase, and alkaline phosphatase were used as specified by the manufacturer. Restriction fragments were analyzed on 0.8% agarose gels in Tris borate EDTA (TBE) buffer (Sambrook *et al.*, 1989).

Transformation of *E. coli* with plasmid DNA

Frozen competent *E. coli* cells were prepared and used as described by Morrison (1979).

Construction of pneumococcus library using cosmid pHC79

E. coli cosmid vector pHC79 is 6.4 Kb and can accomodate approximately upto 44 kb of insert by headful packaging mechanism of lambda phage. Ten μg of chromosomal DNA from CP1200 strain was partially digested with *Sau*3A1. The digested mixture was loaded onto a 10~40% sucrose gradient (bottom: 10% [w/v] sucrose, 1M NaCl, 20 mM Tris-Cl pH 8.0, and 10 mM EDTA) in a 13 mm×51 mm polyallomer tube and centrifuged at 25000 rpm using Beckman SW65K rotor for 12 hours. After centrifugation, 25~40 kb fractions were pooled and dialyzed twice against l liter of 10 mM Tris pH 8.0, 0.1 mM EDTA(TE) buffer. The enriched pneumococcal DNA was precipitated with ethanol and resuspended in TE buffer.

The DNA of the cosmid cloning vector pHC79 was digested to completion with *Bam*H1. Two μg of the partially digested and enriched pneumococcus chromosomal DNA was ligated with 0.5μg of the cosmid vector. The ligated DNA was packaged into lamda particles in vitro using lambda packaging extracts and was infected to DH1 cells as follows.

The clone bank recipient, *E. coli* DH1, was grown overnight in LB broth containing 0.4% maltose at 37℃. Cells were pelleted by centrifugation and resuspended in 0.5 volume of LB broth containing 10 mM $MgSO_4$. The entire mixture of lamda particles containing the recombinant DNA molecules was added to 1 mL of DH1 and incubated for 15 min at 37℃ to allow infection. The mixture was transferred to 5 mL of L-broth and incubated further at 37℃ with aeration for 1 hour

Table I. Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Genotype or relevant marker(s)	References or derivation
E. coli		
DH1	*recA1*	Hanahan, 1983
JM103	*endA sbcB15*	Messing *et al*, 1981
pACYC184 4.0 Kb	Cm-r, Tc-r	Chang and Cohen, 1978
pDKR2030-2062	Tc-r, Cm-s, recombinants	This study
pHC79 6.4 Kb	Ap-r, Tc-r, cosmid vector	Hohn and Collins, 1980
pJDC9 7.0 Kb	Em-r *lacZ'* transcription terminators	Chen and Morrison, 1988
S. pneumoniae		
CP1200	*malM511, str1*	Morrison *et al.*, 1983

to allow expression of the vector encoded ampicillin resistance determinant. The infected cells were plated on LB agar containing 50 ug/ml of Ap and ampicilin-resistant (Ap-r) transformants were used as a pneumococcus library.

Construction of pneumococcus libraries in other *E. coli* plasmids

Endonuclease cut and dephosphorylated vectors were ligated with chromosomal DNA which had been digested with the compatible or same restriction enzyme that was used to create the plasmid digest. Ligation of the DNA fragments into the cloning site of the vector was performed as a typical insert of a DNA fragment into a cloning vehicle. After ligation and transfomation, recombinants in pACYC184 were selected on agar plates containing 15 μg of Tc.

Pneumococcus library construction in pJDC9 was carried out as follows. After ligation and transformation, the transformed JM103 cells were plated in the 3 layered plates containing 1 mg/ml of Em: the top and bottom layers contained 15 ml of B agar with 1.5% agar, and the middle layer consisted of 4 ml of 2X YT agar with 0.7% agar. To the 4 ml of 2X YT agar 15 ml μl of an 0.1 M solution of isopropylthiogalactoside (IPTG; 123.83 mg of IPTG in 1 ml of water), 50 μl of an 2% (w/v) X-Gal (20 mg of X-Gal in 1 ml of N,N'-dimethylformaide) solution, and the transformed cells were added and mixed thoroughly, and poured over the B agar layer. After the 2X YT agar solidified, the 15 ml of B agar (10 g of Tryptone, 8 g of NaCl, 10 mg of vitamine B1, and 15 g of agar per liter) was poured over the 2X YT agar layer and allowed to solidify. The plates were inverted and incubated at 37℃ for 36 hours. Cells containing pJDC9 plasmids with an insert in the polylinker region yielded white colonies whereas cells containing pJDC9 plasmids with no insert yielded blue colonies.

RESULTS

Instability of pneumococcus library in cosmid pHC79

Wild type chromosomal DNA was partially digested with *Bam*H1 and 25~40 Kb DNA fragments, collected after fractionation in 10~40% sucrose gradient, were ligated to pHC79 vector which had been cut by *Bam*H 1. The ligated DNA was packaged into lambda particles *in vitro* using lambda packaging extracts, and was infected to DH1 cells. The resulting transformants were selected on Ap plates and 849 Ap-r colonies were collected. But when they were transferred to the second plates containing 50 ug/ml of Ap for colony purification, most of them did not grow. Only few colonies, less than twenty, grew very poorly. When the few colonies were transferred again to the third plates containing Ap, all of them except two were grown. DNA structure of those two stable colonies were examined by restriction enzyme and found that they contained 30 Kb and 37 Kb insert, respectively(Table II). This result suggested that most of the recombinants containing large inserts of pneumococcal DNA became unstable and/or degenerated rapidly.

Low efficienciy of pneumococcus library construction in pACYC184

To avoid instability of pneumococcus library in *E. coli* cosmid vector, I tried to make a pnuemococcus library containing smaller insert(s). First, pneumococcus DNA partially digested with *Sau*3A1 was fractionated by 10~40% sucrose gradient and 7~40 kb fractions (average 20 kb) were ligated with the vector pACYC 184 which had been digested with *Bam*H1 and dephosphorylated. Cm-r, Tc-s clones which had insertional inactivation of Tc-r determinant at *Bam*H1 site were selected as recombinants and DNA structures were examined by restriction enzyme digestion. Table III shows that 34 out of total 115 clones had the recombinants structure smaller than the size of original vector (4 kb). And 50 out of 115 had inserts smaller than 1 kb. Only 3 of them were shown to have recombinat structures bigger than 10 kb.

Instability problem was also observed when pneumococcus library was constructed by *Eco*RI digestion followed by ligation with the pACYC184 cut with the same restriction enzyme. Cm-s, Tc-r clones which had insertional inactivation of Cm-r determinant at *Eco*RI

Table II. Shotgun cloning of pneumococcal DNA in cosmid pHC79

Pneumococcal DNA[a]	vector	stability[b]	plasmid structures in stable transformant
*Sau*3A1	pHC79	847	
partial	cosmid	unstable	(1) 30 kb insert, 5 *Eco*RI sites
sucrose:		2	
6~40 kb	*Bam*H1	stable	(2) 37 kb insert, 6 *Eco*RI sites

[a] Partial digestion of the chromosomal DNA from wild type strain was performed as described in the Method section.
[b] Stable transformants were defined as the transformants which could grow well when transferred from the initial selection agar plate to the second selective agar plate for colony purification.

Table III. Shotgun cloning of pneumococcal DNA in *E. coli* vectors

Pneumococcal DNA[a] structures[d]	Vector[b]	Yield[b]	Stability[c]	Number of stable transformants with indicated plasmid					
				< 0 kb	< 1 kb	1~2 kb	2~5 kb	5~10 kb	> 10 kb
*Sau*3AI partial sucrose 7~40 kb	pACYC184 *Bam*HI dephos-phorylation	50% Insertion	all stable Insertion	34	50	17	8	3	3
				< 0	< 1 kb	1~3 kb	3~5 kb	5~10 kb	> 10 kb
*Eco*RI partial avg 15 kb	pACYC184 *Eco*RI dephos.	96% Insertion	all stable	6	19	7	6	2	3

[a] Partial digestion of the chromosomal DNA from wild type strain was performed as described in the Methods section.
[b] The yield of recombinant was defined by the ratio of recombinants/total transformants. If transformants showed insertional inactivation of drug resistance marker or if plasmid DNA in the transformants had insert, they were counted as recombinants.
[c] Stable transformants were defined as the transformants which could grow well when transferred from the initial selection agar plate to the second selective agar plate for colony purification.
[d] Plasmid structure was determined by electrophoresis on 0.8% agarose gels on *Bam*H1 digests of small-scale plasmid extracts of selected Cm-r, Tc-s transformants. About one third of plasmids lost *Bam*H1 site and, therefore, they were digested with *Eco*RI to determine linear fragment size. The size of pACYC184 is 4.0 kb and recombinant plasmid less than 4 kb was considered as deleted recombinants.

Fig. 1. Physical maps of pHC79, pACYC184, and pJDC9. pHC79(Hohn and Collins, 1980) contains Ap-r and Tc-r determinants, those genes are indicated by insertion at Pst1 and BamH1, respectively. The exact length of lambda DNA is not known. pACYC184 (Chang and Cohen, 1978) contains Cm-r and Tc-r determinants and those genes are inactivated by insertion into the *Eco*RI and *Bam*H1 sites. pJDC9 (Chen, 1987) contains a multiple cloning site surrounded by tandemly repeated *E. coli rrnB* terminators (T_1T_2) and λ *oop* RNA terminator (t_0). The multiple cloning site contains 10 unique restriction sites; *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sph*I, *Sst*I, and *Xba*I.

site were selected as recombinants. Recombinant DNA structure in *Eco*RI library showed almost the same pattern as the *Sau*3A1 library. Six out of 43 clones showed recombinant structure smaller than the vector, and 19 clones had insert smaller than 1 Kb. Only 3 clones had recombinant structures larger than 10 Kb. Fig. 2 demonstrated typical deletion and/or rearrangement of pneumococcal inserts in pACYC184 *Eco*RI site. This result demonstrated that most of the recombinants lost the pneumococcal inserts and the *Eco*RI site of pACYC184, and became smaller than the vector.

High efficiency of pneumococcus library construction in pJDC9

Because most of the recombinants constructed in cosmid pHC79 and pACYC184 had been unstable, a plasmid vector which could give rise to stable recombinants was seeked. Considering the fact that pneumococcal DNA fragments have strong promoter activity in *E. coli* (Prats *et al.*, 1985; Stassi and Lacks, 1982; Martin *et al.*, 1985) and require transcription terminator vector (Chen and Morrison, 1987), a plasmid vector pJDC9 containing transcription terminators was emplo-

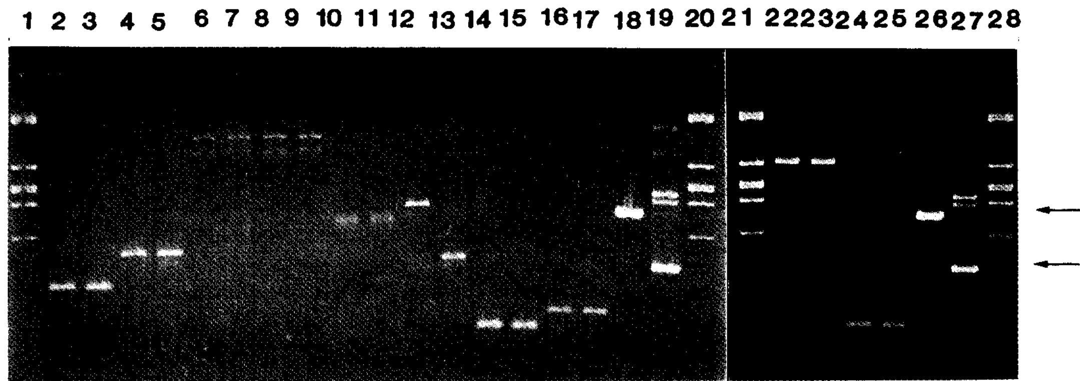

Fig. 2. Deletion and/or rearrangement of pneumococcal inserts in pACYC184. 7~40 Kb CP1200 chromosomal DNA, pooled after partial digestion with EcoR1 and fractionation on sucrose gradient, was ligated to pACYC184 which had been digested with *Eco*RI and dephosphorylated. After ligation and transformation, plasmid structure of the resulting Tc-r and Cm-s transformants (named pDKR2030 through pDKR2062) were examined by EcoR1 digestion followed by a 0.8% agarose gel electrophoresis. Upper and lower arrows indicate a linearized 4 Kb pACYC184 DNA and a undigested supercoiled pACYC184 DNA, respectively. Lane 1, 20, 21, and 28, lambda DNA digested with *Eco*RI. Even numbered lanes are digested with *Eco*RI and odd numbered lanes are not digested: lanes 2 and 3, pDKR2042; lanes 4 and 5, pDKR2041; lanes 6 and 7, pDKR2040; lanes 8 and 9, pDKR2039; lanes 10 and 11, pDKR2038; lanes 12 and 13, pDKR2037; lanes 14 and 15, pDKR2036; lanes 16 and 17, pDKR2031; lanes 18 and 19, pACYC184; lanes 22 and 23, pDKR2062; lanes 24 and 25, pDKR2043; lanes 26 and 27, pACYC184.

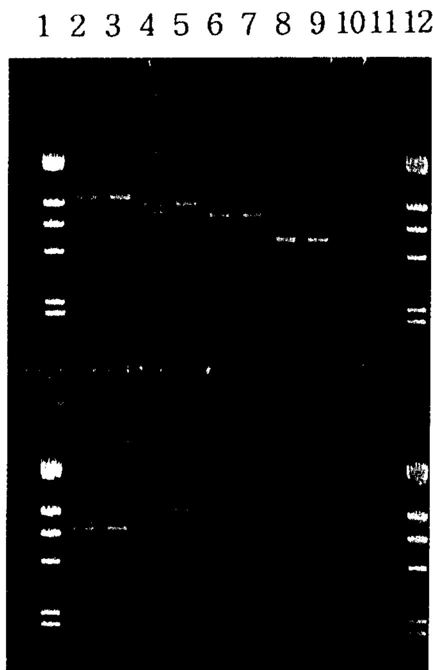

Fig. 3. Recombinant DNA structure of pneumococcus inserts in pJDC9. 5~15 Kb CP1200 chromosomal DNA, pooled after partial digestion with *Pst*I and fractionation on sucrose gradient, was ligated to pJDC9 which had been digested with *Pst*I and dephosphorylated. After ligation and transformation, plasmid structure of the resulting Em-r, white transformants were examined by *Pst*I digestion followed by a 0.8% agarose gel electrophoresis. Lanes 1, 12, lambda DNA digested with *Hind*III; recombinant DNA isolated from Em-r white transformants undigested (lanes 3, 5, 7, 9, 11) and digested with *Pst*I (lanes 2, 4, 6, 8, 10, respectively).

yed for construction of a pneumococcus library. 5~15 kb of pneumococcus DNA digested with *Pst*I and fractionated by electroelution was used for the construction of pneumococcus library in pJDC9. Recombinants in pJDC9 were found stable and none of them was found to lose the insertion site (Fig. 3).

DISCUSSION

Pneumcoccus library constructed in cosmid vector was extremely unstable that only 2 clones were stable. Similar instability problem was also observed in pneumococcus library constructed in pACYC184. But the frequency of unstable recombinants was much lower in pACYC184 pneumococcus library than that of cosmid vector. This result suggests that pneumococcus library containing large insert is much more unstable than the pneumococcus library containing small insert. But this instability problem seemed to be resolved by using the transcription terminator vector pJDC9. When small insert was used for construction of pneumococcus library, similar phenomena was observed; When 3~6 kb of pneumococcus DNA was shotgun cloned in pUC19, no recombinants were found to have intact inserts. Only 17 out of 69 transformants had intact insert of 1~3kb (Chen and Morrison, 1987). But this instability problem might be rare if the pneumococcus insert size is smaller than 2 Kb (Dillard and Yother, 1991).

Instability of pneumococcus genes in *E. coli* vectors was also observed by others (Prats *et al.*, 1985; Chandler and Morrison, 1987; Chen and Morrison,1987; Stassi

and Lacks, 1982; Martin *et al.*, 1985, 1989). Stassi and Lacks (1982) showed that the pneumococcal *mal* region exhibited strong promoter activity and interfered with maintenance of a recmobinant plasmid in *E. coli*. And cloning of *mal* region in pBR322 was stabilized by a down-mutation in that promoter. The pneumococcal *com* locus was unstable in pMB9, but it was stable when cloned in the terminator vector, pKK232-8 (Chandler and Morrison, 1987). And both *mal* and *com* loci showed strong promoter activity in *E. coli*. Thus the instability of pneumococcus gene library in *E. coli* seemed to be, at least in part, due to the strong promoter activity from the pneumococcal insert. This suggestion is supported by the fact that pneumococcus DNA has high AT content which can act as promoter in *E. coli* (Morrison and Jaurin, 1990). But, at this stage, it is too early to draw conclusion. Several explanations may account for the instability of the pneumococcus library in *E. coli*; 1) toxic gene product, 2) some problems unrelated to promoter strength such as reductions in conformational changes that could result in deletions, or 3) strong promoter activity. Further genetic studies on pneumococcal genes will provide answer for the reason of instability.

ACKNOWLEDGEMENT

This work was supported in part by a grant from Korea Science and Engineering Foundation (911-04 07-030-2).

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