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Partial Sequencing of Ampicillin Resistant Plasmid from Klebsiella

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INTRODUCTION

The 13-lactam antibiotics: Soon after the introduction of penicillin for clinical use, it was recognised that organism could develop resistance to penicillin. During the irst twenty years which followed the clinical use of penicillin, development of new compounds based on the penicillin nucleus took place slowly, but in the twenty years from 1 960 to 1 980 many different types of penicillin became available. Yet the organisms developed resistance to 13-lactams by different mechanisms such as reduced cell permeability, reduced target affinity, and modification of the antibiotic. Of these the modification of antibiotic by P-lactamase production is an important mechanism of resistance development (Georgopapadakou, 1993).

Plasmid or transposon encoded 13-lactamases: The enzyme 13-lactamase catalyzes the opening of the (3-lactam ring and may be encoded by the bacterial chromosome or by plasmids. However, the plasmid or transposon mediated 3-lactamases bear little resemblance to the chromosomal type present in clinically important species (Mediros.1984). The spread of transposon-borne 13- lactamases in S. aureus has greatly reduced the anti Staphylococcal eficacy of benzyl penicillin. Thus in 1 940 less than 1% of the isolates were found to have lactamase and by 1960 the presence of this enzyme was detected in about 70-90% of the isolates The introduction of methicillin and isoxazolyl penicillins, which are not acted upon by the Staphylococcal penicillinase overcame the problem presented by this enzyme at least tentatively. The major concern however, is not only the increase in the number of various plasmid-mediated 13-lactamases but their spread to P.veudomonas acruginosa, Hinfluenzae and N.gonorrhoeae (Sykes and Matthew, 1976). Other common plasmid-mediated 13-lactamases include SH V-1, which frequently occurs in Klebsiella (Kitzis et al 1 988) and PSE-1 and PSE-4 which are largely restricted to Pseudomonads (Medeiros et al 1982). Earlier it was observed that plasmid-mediated fl-lactamases of gram negative rods cause resistance to a-amino and a-carboxypenicillins and older cephalosporins other I "than cefamandole and cefoperazone but not to temocillin, imipenem or most newer cephalosporins (Jacoby and Sutton. 1985). However, recently new types of plasmid mediated cephalosporinase have evolved from the TEM-type enzyme. These new enzymes also cause resistance to most second and third generation cepholosporins (Sutton et al. 1991; Medeiros et al. 1 995; Heym et al. 2000).

The use of plasmid mediated transformation or conjugation experiments involving the donor Klebsiella aerogens (VM008) and the recepient E coli produced only ampicillin resistant transformants / transconjugants. Therefore, a characterization of one of the ampicillin resistant plasmids obtained in the transformation experiment was undertaken to determine its salient features.

MATERIAL& AND METHODS:

Materials:

The , restriction enzymes were purchased from Boehringer Mannheim.Germany, Sequencing. kit (sequenase vertion2.0) was purchased from United States Biochemicals (USB). Sequesing grade acrylamide, urea, ammonium persulphate TEMED was purchased from SIGMA chemicals. USA. Radioactive a-35[S] d ATP and hypertilm was purchased from Amersham.

METHODS:

Construction of restriction map of the ampicillin resistant plasmid: To construct the restriction map, plasmid (designated pVMRF 8.6kb, which was obtained by transforming E.coli using the plasmids isolated from K aerogens), was digested with different restriction enzymes. Plasmid DNA (I and 51.1.1 of the buffer and 5units of the respective enzyme in a 50111 of reaction volume was incubated at 37`)C for I hr. After incubation,

samples were subjected to agarose gel electrophoresis with the appropriate marker. The enzymes that have unique sites on the plasmid were identified. The enzymes BamH I, Sal I, Cla I and Sma I were found to have linearizing pVMRF of size 8.6kb. The locations of these unique sites relative to one another were then determined by digesting the plasmid with two different enzymes and the relative fragment size obtained was recorded. For example, to know the position of the BamH I site with respect to other unique sites, the plasmid was first digested with BamH I and the linear plasmid obtained was used for the digestion using a second enzyme. The fragments obtained after digestion with each set of enzymes were noted after agarose electrophoresis. Sites for several other enzymes having more than one restriction site were also identified by digesting • the plasmid and analyzing the products by gel electrophoresis.

Sub cloniig of the plasmid: With the information generated from the mapping of restriction sites on the plasmid pVMRF, the various clones were arranged in orderly fashion from one direction of the linear map of the plasmid pVMRF in a total of eight subclones (clone Ito clone 8 of the sizes I .0Kb, 1 .2Kb, —0.6Kb, I.9Kb, 0.9Kb, 0.6Kb, 0.8Kb, I .5Kb). After the restriction digestion, samples were loaded on low melting agarose gel and DNA bands were cut out and purified by Clean Genei kit (explained in Chapter 111).

Ligation reaction: A gel-purified fragment (60ng) was used for the ligation reaction with the vector DNA (pUC 1 8) digested with the same restriction enzyme. Vector to insert ratio in the ligation reaction was 1 : 3 in a total ligation reaction volume of 204 The T4 DNA ligase (I unit) was then added to the reaction mix and incubated overnight at 16°C.

Transformation: After overnight incubation, the ligation mix was used to transform E.coli NM 522 by heat shock method as explained in (the earlier section). After transformation, colonies appearing on the LB agar+ampicillin plates were screened for recombinant clones. Large-scale purification of the plasmid from these clones was done by using alkaline lysismethod.(Maniatis, et al. 1 995).

Sequencing of the subclones:

Denaturation of template DNA:

The sequencing reaction was carried out according to the manufacturers instructions (sequenase version 2.0,USB). The template DNA (2pg) was denatured by adding NaOH (final concentration of 0.2 N) in a 20p1 final volume and incubated at 37°C for 20min. The I/10h volume of 3M sodium acetate was then added followed by 2.5 volumes of absolute ethanol. The DNA was precipitated by keeping the mixture at —70°C for I hr.

Thereafter it was pelleted by spinning at 1 5000 rpm for 10min in a microfuge.

Annealing reaction:

The denatured template DNA (lpico mole) was taken and mixed with 0.5 picomoles of universal primer to which 21.1.1 of annealing buffer was added. The final volume was adjusted to 1041 by adding water. The tube was heated to 65°C for 2min and the mixture was allowed to cool slowly to the room temperature over a period of about 30min and then chilled on ice.

Labelling reaction:

To the annealed primer, 11.11 of 10mM DTT, 2121 of labelling mix and 512Ci of a-35[S] dATP (supplied by Amersham International) and 2units of sequenase enzyme (1:8 fold diluted in enzyme dilution buffer supplied in the kit) was added. The components were mixed thoroughly and incubated for 5min at room temperature.

Termination reaction:

The appropriate dideoxy termination mix (2.5i11) was added to four corresponding tubes labelled A,G,T and C. After the labelling reaction was completed, 3.411 of the labeled mixture was added to each of the four tubes and incubated at 37°C for 5min. Following this, stop solution (41.11) was added to each of the four tubes and stored on ice.

Electrophoresis:

The glass plates were cleaned and siliconized, rinsed with distilled water and dried. The plates were assembled with 0.4-mm spacers. To 10m1 of 10X TBE buffer, 7.6gm/0.4gm acrylamide/bis-acrylamide combination, and 42g of urea were added, and after solubilization, the volume was made up to 100m1 with milli Q water. This mixture was then filtered and degassed for 10min. To this lml of 10% ammonium persulfate and 25ml of TEMED were added, swirled gently and immediately poured between the glass plates. The plates were laid horizontally and sample application combs were inserted at the top of the plates. The assembly was then left for an hour to allow the gel to polymerize. The gel was then mounted on electrophoresis apparatus (BRL) and the lx TBE butler was added to the upper and lower tanks.

The gel was pre run at constant power of 40W for 45min. Ater this pre run 2p1 of denatured (at70°C) sequencing reaction mix was loaded in to adjacent wells in sets of four

(like A,G,T,C). The same sets of samples were loaded in adjacent wells two more times at an interval of two hours. Electrophoresis was continued until the xylene cyanol in the most recently loaded set was 5 cm from the bottom of the gel.

The gel was then transferred carefully to a supporting sheet of filter paper covered with high quality plastic wrap and then dried using a vacuum gel drier at 70°C. The dried gel was then exposed to Hyperfilm (Amersham) for 16hrs and then developed. The sequence was read from autoradiogram.

DNA SEQUENCE ANALYSIS:

Plasmid sequence obtained was analyzed using the BLAST search to get the homology regions against the GENBANK database at Bioinformatics Division, Indian Institute of Science.Bangalore. This program and its default parameter settings were optimized to find nearly identical sequences rapidly (Lipman, et al. 1 990)

RESULTS AND DISCUSSION:

Construction of restriction map of the ampicillin resistant plasmid: To construct a map of the sites at which restriction enzymes cleave DNA, it is usually necessary to employ more than one restriction enzyme to obtain map that is sufficiently accurate and detailed to be useful. From the size of the DNA fragments generated in the enzymatic digestion, it is usually possible ,to deduce the relative locations of at least some of the cleavage sites. As the number of pair wise combinations of enzymes increases, the position of restriction sites can be more accurately assigned. The correctness of the map constructed depends very much on the accuracy with which the sizes of the DNA fragments are determined using the markers.

To construct the restriction map of pVMRF, enzymes having a single restriction site on the plasmid were identified. The digestion of the plasmid by Barn HI, Cla 1, Sal I, or Sma I gave rise to a single band of about 8-9kb size in agarose gel electrophoresis (Fig.4.0.Lanes.2, 5, 8 and 11 respectively). Thus, all these four enzymes appeared to have a unique restriction site. The position of a unique site on the plasmid with respect to another unique restriction site was decided based on fragments generated by two enzymes. For example, the digestion of the plasmid with BamH I and Cla I resulted in the production of linear fragments of the sizes 0.6kb and —8kb indicating that these two sites are nearly 600 bases apart. Similarly digestion of the plasmid with BamH I and Sma I gave rise to a —3.5kb and a —5kb fragment indicating that these two unique sites are 3500 bases apart. Another unique site Sal I was situated —l .5kb apart from the BamH I site as indicated by the fragment released upon digestion by these two enzymes. The digestion of the plasmid with Sal I and Sma I resulted in the release of a fragment sizes —0.7kb and a —8kb indicating that the Sma I and Sal I sites are —700 bases apart. The digestion of the plasmid with Cla I and Sal I released a fragment of —2.2kb size. This released fragment upon digestion with Bam HI produced two fragments of 1.6kb and 0.6kb sizes. This clearly established the location of Ban HI site. Similarly, the fragment obtained by digesting the plasmid with BamH I and Sma I showed the presence of Sal I site within.

The restriction positions of the other enzymes having more than one site on the plasmid was decided with respect to the unique sites after a series of restriction digestions. The digestion of the plasmid with Bgl 1 resulted in the production of the fragments of the sizes 3.5kb, 2.5kb, 1 . 5kb and 0.9kb indicating the presence of four sites on this plasmid (Fig.4.0.Lane3). The enzyme Bgl 11 produced two fragments of the sizes 2.2kb and —6kb indicating the presence of two sites (Fig.4.0.Lane4). The digestion of the plasmid with Pst 1 enzyme gave four fragments of the sizes 2.9kb, 2.7kb, 2.2kb and 0.6kb ther by indicating the presence of four sites (Fig.4.0.Lane6). The enzyme Hind III generated two fragments of —2.5kb and —6kb (Fig.4.0.Lane7) sizes indicating the presence of two sites on the plasmid. The use of enzyme Ava II resulted in the production of four fragments —3kb, 1 .9kb, 1.2kb and 1 kb sizes that indicated the presence of at least four sites (Fig.4.0.Lane9). However, the sizes of the fragment obtained with the enzyme Ava II digestion do not correspond to the estimated size of the plasmid. This suggests that some of the Ava 11 sites are very close to each other. Therefore, the position of the Ava 11 sites on the plasmid was assigned with respect to the best possible resolution of the fragments obtained with other sets of enzyme digestions. The presence of two sites for Pvt, II was indicated by the production of two bands: a small 300bp fragment and a large 8kb fragment (Fig.4.0.Lane10). The positions of restriction enzymes having more than one site were decided using a series of internal digestions with one or more enzymes. The positions of these restriction enzymes on the plasmid are given on the circular map (Fig4.1).

Subcioning of the plasmid pVMRF in to pUC18 for sequencing: Since a similarity in the Pst I restriction digestion pattern in the plasmids isolated from transformants was observed, it was felt that sequence information of one of them would give an idea of the nature of the plasmid.

Sequence homology found in the GENBANK database: The total size of the plasmid pVMRF was determined to be —8.6kb. The eight sub clones of this plasmid were arranged in order starting from one of the Pst I sites in an

anti clock wise direction. One of the Pst I restriction sites was marked as the start off point to facilitate description. The restriction site for Hind III was I .5kb away from this Pst I site and this constituted the clone no 8. This was followed by other clones that were numbered in descending order (7 to I). The coordinates for each subclone and the partially sequenced area in each clone are given in Fig.4.4b.

The sequences read from each clone were analyzed for open reading frames (ORFs), and the amino acid sequences deduced from the ORFs were compared with the sequences of known polypeptides in the database.

The origin of replication:

. "A comparison of the sequence obtained from clone 8 revealed that it is (90%) homologous to the E coli plasmid p 1 5A fragment specifying the origin of replication (Selzer, 1983). The sequences read from clone no.8 comprised the start codon of the protein Rom (RNA 1 modulator) with the ribosome binding site. The oriV region, the point of replication initiation was also observed. The stop codon for the protein Ram was seen in the sequences obtained from clone no 7 (Fig.4.5) suggesting contiguity. The sequences from pVMRF showed complete homology to the replication region of the plasm id pTKH I lisolated from K oxyloca (Wu, el al. 1 999). Replication of Col El-related plasmids is controlled by the binding of RNA I to RNA II. In some plasmids, this interaction is regulated by the product of the ram gene. In ColE1, ram maps immediately downstream of oriV and encodes a small acidic protein, which enhances the inhibitory activity of RNAI in vitro and in vivo. This effect is mediated through Rain-dependent stabilization of the RNAI-RNAII complex (Cesarini,a a/.1991). The rom gene product is reported to be not essential but exerts some control over plasmid copy number. This replication control region has also been reported in plasmids isolated from K pneumoniae (Tolmasky, 1 997) and plasmids isolated from Salmonella enierica (Keenleyside, et a/.1995). The sequences read from clone no 7 showing the presence of stop codon for Rom and start codon for the protein Excl 1 are given in the Fig.4.5. The sequence alignment of the plasmids pVMRF and pTKH11 are given below. In addition, the upstream sequences of the Rom, which includes the ribosome binding site and the origin of replication oriV, are also shown.

Following the stop codon of Rom protein the reading frame for the entry exclusion protein 1(Excll) was found. The direction of transcription for Excll appears to be in the same direction as that ofRom protein. The sequences found in pVMRF showed complete homology to the sequences found in the pTKH11 except the 27 amino acids (position 57 to 83) in the 3" region of the gene.

This gene product has been reported to be involved in confering resistance to the conjugal transfer of DNA between mating pairs carrying homologous ColE1 plasmids (Chan, et cd.1985). This Excil protein was also reported to be present in a 5kb plasmid pKlebBk17/80 isolated from K pneumoniae (accession no NC002610). The comparison of the sequences fot the protein Excl 1 is given below.

Ampicilin resistance gene:

The sequence information obtained from the plasmid of clone no 6 revealed the presence of an extended spectrum of f3-lactamase gene. The 312-nucleotide sequence representing the 3" region of the gene beta lactamase showed a 100% match with the sequences of (1) TnSFI found in a 26kb plasmid isolated from Shigella flexneri (accession no.

In the present study, although the C-terrninal region was identiied, the N-terminal region was not found in the limited sequenced region. The amino acid positions 181 to 286 match perfectly with the sequence found in the plasmid pJD4 (nucleotide region 1-320) (Dillon, et a/. 1999; Pagotto, et al. 2000). This part of the sequence has also been found in the inhibitor resistant P-lactamase from E colt isolates (Leflon, et al. 2000). The 312 nucleotide constituting carboxy terminal 104 amino acids of fl-lactamase found in pVMRF from clone no 6 are given in the Fig.4.6. Thel3-lactamase gene sequence found in pVMRF is aligned with plasmid pJD4 sequence.

The plasmid-encoded TEM enzymes are the most prevalent j3-lactamases in Enterobacteriaceae (Mediros, 1986). TEM-1 and TEM-2 are found in a substantial majority of clinical isolates (Ambler, 1979). Transferable resistance to broad-spectrum cephalosporins has been described in the species of Klebsiella (Kliebe, 1985; Knothe, 1983; Shah, 1983; Sirot, 1987; Petit, 1988). The p-lactamse of the TEM type shows large variations in some of the amino acid residues. The microbes have evolved different 13-lactamases through altering certain specific amino acid residues to enable their catalytic potential towards new generation of 13-lactams. The analysis of the sequences of various 13-lactamase show that the highest frequency of naturally occuring mutations are at positions 104, 164, 238 and 240 (Table 4.0). Interestingly the results of directed evolution experiments have also indicated that these residues are most likely to get altered in the natural selection process. In 1994, experiment carried out using directed evolution technique predicted that a combination of mutations in the 13-lactamase would occur leading to E->K, M->T, and G->S substitutions at positions 104, 182, and 238. It is remarkable that a b-lactamase with precisely the predicted changes was found in a clinical isolate (Orencia,

et al. 2001). In the present study, the fl-lactamase found has changes at multiple locations leading to the formation of E104S, M182A, and G238R.

Moreover, changes at positions 164, 238, 240 and 265 were also observed. Combination of mutations occuring at positions 164 and 238 are considered deleterious (Orencia, et al. 2001). In one instance however, changes such as R164S and G238S have been observed but only in the presence of stabilizing mutation at position 104 resulting in the ubstitution of aspartic acid with lysine (Jacoby and Bush.http://www.lahey.org/studies/webt.htm; Gilbert, et a1.2001) Thus, there seem to be a formation of distinct p-lactamase in the Klebsiella isolate. The spread of such an altered vertion of the enzyme could pose a serious problem for drug design.

The 13-lactamase amino acid sequence deduced from the partial nucleotide sequence obtained from pVMRF was analysed further using GCG software. Since the carboxy terminal 312 nucleotides are homologous to the sequence found in the plasmid pJD4, for, this analysis the N-terminal sequences were taken from the sequences found in that plasmid. The complete polypeptide sequence (286 amino acid) was subjected to peptide structure analysis. Using GCG software, the secondary structure was predicted. Features such as hydrophobicity, and surface probability were predicted, and the results are presented in the pepplot.(Fig.4.7). The charge as a function of pH for peptide sequence was predicted using this software and the predicted pl value of this peptide is 5.95 (Fig.4.8.). The pi value of the TEM I enzyme is 5.4. The secondary structure analysis of this peptide did not show significat difference in its structural features when compared with the TEM I sequences. The change in the amino acid composition of this peptide does not seem to alter its structural features. However, the changes observed in different amino acid positions in this peptide that may alter its structural specificity in the spectrum of antibiotics need to be studied further.

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