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Bacterial Plasmids

The invention relates to bacterial plasmids.

Plasmids are small, extra-chromosomal, mostly circular and independently replicating DNA molecules, which occur in nearly all bacteria and also in some eukaryons as well as in the mitochondria. The size of the plasmids vary between approximately 1,5 to 300 kb.

As a rule bacterial plasmids are circular, covalently closed and supercoiled. They often carry resistance genes against antibiotics or heavy metals, genes for the metabolization of untypical substrates or genes for a number of specie-specific characteristics, such as metabolic properties or virulence factors. Some plasmids can be transferred from one cell into another. Because the pathogenity of bacteria is determined according to present views partly also by the properties of the plasmids, there exists increasingly a greater interest to clarify the properties of the plasmid DNA.

It is known of the family of entereobacteriacea, to whom 14 main varieties and 6 further varieties belong, that these can develop different properties. Typical examples are Escherichia, Salmonella and Klebsiella. Escherichia coli (E.coli) is the classic object of bacteria genetics. Only on discovering and characterizing different virulence factors of E. coli it was made it possible to find general satisfactory explanations therefor that strains of this species exhibit differences in human and animal pathogenities, which are partly extreme and extend from avirulence to high grade virulence, such as in the case of the recently spreading variant known as "EHEC". Thus a number of virulence factors have already been described for extra-intestinal as well as for intestinal E. coli strains, which partly have been excellently

characterized. For pathogenic E. coli strains the sero-variety O6:K5 virulence factors, such, as for example, haemolycin and P-fimbrial adhesion were found, which have been found not to appear at apathogenic representations of this sero-variety.

As a rule virulence genes are found at enterobacteria on large plasmids (approximately 60 kb). There are also enterobacteria with small, so-called cryptic plasmids, whose function hitherto could not be reliably determined.

Because it is known that in the case of E. coli virulence factors are at least partly also present in the genes of the plasmids, there therefore exists a need for further investigation regarding the detection and characterization of plasmids at enterobacteria and especially Escherichia, to improve, for example, the diagnostic and therapeutical possibilities of illnesses after infections with Enterobacteria. The plasmids or their bacterial carriers or corresponding synthethized DNA can be applied medicinally in the therapy or prophylaxis and also for nutritional physiological or probiotic purposes in the microbiological analytics or diagnostics. Furthermore there are plasmids, and in fact especially those of E. coli, known expression vectors in the genetic engineering, so that also for this reason an interest exists in an improved knowledge of the properties of such plasmids.

Molecular genetic investigations with the E. coli strain DSM 6601 were performed for this purpose. The DNA sequences obtained from this strain were examined with the help of data bank programmes of DNA sequence analysis and compared with already existing DNA sequences of other bacteria.

The strain DSM 6601 contains two small plasmids with a size of respectively 3177 or 5552 bp, which are described respectively as pMUT1 or pMUT2.

The DNA of the smaller plasmids pMUT1 were sub-cloned as a linear fragment into the vector pUC18, after restrictional fission with the enzyme HindIII, the DNA sequence was subsequently determined. This is evident from the enclosed Figure 1. The resulting DNA sequence was examined by means of a homologous comparison with the GenEMBL data bank; the results of this comparison are given in Figure 2.

For this comparison the HindIII intersection was fixed as Position 1. From the position 200 to 800 bp the DNA of the plasmid pMUT1 exhibits significant homologies to different reduplication starting positions (origins of replication) of other enterobacterial plasmids, especially to the plasmids NTP1, NTP16 and CloDF13. In the region of position 950 bp a 570 bp sized homology starts to the plasmid NTP16, which originally was isolated from Salmonella Typhimurium. This homology contains the mobA-gene, which is necessary for the mobilizability of the plasmids NTP16 as well as for an origin of transfer (oriT). Furthermore, significant homologies to the gene parA and cer were found from position 1790 up to 1920 bp, which are significant for the stability and the continuous transfer and distribution of plasmid molecules during the cell division. For the remaining DNA regions no significant homology could be identified.

Furthermore the DNA sequence of the plasmids pMUT1 were transcribed into an amino acid sequence and analyzed for the existence of open reading frames. Six different reading frame possibilities were investigated. In total 5 open reading frames with a size of 143, 62, 56, 49 and 48 amino acids could be found. A graphic representation of the analysis is given in Figure 3.

The DNA of the larger plasmid pMUT2 were likewise sub-cloned after linearization with the restriction enzyme SphI into the vector pUC18 and subsequently were sequenced completely. The DNA sequence is given in Figure 4. The DNA sequence obtained in this manner was investigated with the help of the GeneEMBL data bank programme for homologies with already known DNA sequences. The result is graphically represented in Figure 5. The DNA of the plasmid pMUT2 exhibits significant homologies to the replication region of different ColE1 plasmids of E. coli in the position 890 up to 1660 bp. A further significant homology to ColE1 plasmids is found in the region of position 3800 up to 4950 bp. Here it concerns homologies for the mobilization region of ColE1 plasmids. In the area of position 3770 up to 4980 bp homologies were found to a plasmid of the Pasteurella Haemolytica strain A1. There are genes on this plasmid, which encode at Pasteurella for anti-microbial resistance proteins. However, the homology stretches over the intergenetic region, so that it possibly also concerns sequences, which are necessary for the mobilizability of the plasmids.

Two regions were identified, which exhibit significant homologies to other enterobacterial plasmids. Thereby it concerns the origin-of-replication-regions and mob-regions, which are necessary for the mobilizability of the plasmids. For pMUT2 no significant homology could be identified for the remaining DNA sections.

Also the DNA sequence of the plasmid pMUT2 was subsequently circumscribed into an amino acid sequence and analyzed on the existence of open reading frames. The result is likewise graphically represented in Figure 3. Five open reading frames with amino acid sequences in the order of 327, 318, 264, 76 and 63 amino acids were found.

Besides the hitherto unknown plasmids pMUT1 and pMUT2, their combination was hitherto found in no E. coli strains or other enterobacteria.

The occurrence of plasmids stands possibly in relation to the metabolical and medicinal and/or nutritional physiological or probiotic usable properties of the strain DSM 6601.

The investigation of the plasmids allows a more precise determination and analysis of enterobacteria and especially the Escherichia groups. Furthermore these plasmids offer themselves as reliable expression vectors for genetic engineering.

The invention shall now be further explained by way of examples:

Example 1:

Plasmid isolation.

The isolation of the plasmid DNA occurred according to the process of Birnboim et al. (Birnboim, A.C. und Doly, J. (1979) Nucl. Acids Res. 7:1513-1523 A rapid alkaline extraction procedure for screening recombinant plasmid DNA).

3 ml LB-medium were inoculated with a bacteria colony and agitated overnight at 37° C. This culture is centrifuged off in an Eppendorf vessel, the medium residue is removed by means of a pipette. The cell sediment is re-suspended with 100µl of solution I (50 mM glucose; 10mM EDTA, pH 8; 25 mM Tris-HCl, pH 8). After 5 minutes of incubation at room temperature 200 µl of solution II (0,2 N NaOH; 1 % SDS) is added, mixed until clarification and the Eppendorf vessel is left on ice for a further 5 minutes. Then 150 µl of solution III (3 M Na-acetate, pH 4,8) is added thereto, agitated briefly until the chromosomal DNA precipitates flocculantly, and the sediment is left again on ice for 5

minutes. The precipitated chromosomal DNA and the cell residues are pelletized for 5 minutes in the centrifuge and the residue with the plasmide DNA is transferred to a new vessel. For the purification of the plasmid DNA, 50 µl phenol and 150 µl chloroform/isoamyl alcohol (24:1) are added and after brief agitation centrifuged off for 2 minutes. The aqueous phase is pipetted into a new vessel. The plasmid DNA is precipitated with 2 volumes ice-cold ethanol and centrifuged off for 10 minutes. The pellet is washed with 70 % ethanol and dried in the Speedvac. The plasmid DNA is re-suspended in 20 µl H_2O_{bidest} and preserved at -20° C.

Example 2:

DNA sequencing

The DNA sequencing occurs according to the process of F. Sanger et al. (Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467 DNA sequencing with chain terminating inhibitors).

The DNA sequencing occurs with the T7-sequencing kit of the company Pharmacia LKB.

For the denaturization step 8 µl (1,5 up to 2 µg) plasmid DNA is mixed with 2 µl 2 N NaOH, briefly centrifuged off and incubated for 10 minutes at room temperature. The DNA is precipitated with 3 µl 3 M Na-acetate, pH 4,8 as well as 7 µl H_2O_{bidest} and 60 µl ice-cold $ethanol_{absolute}$ for 15 minutes at -70° C. The precipitated DNA is centrifuged off for 10 minutes, washed with 70 % ethanol and dried.

For the annealing reaction the denaturated DNA is suspended in 10 µl H_2O_{bidest} and mixed with 2 µl annealing powder and 2 µl primer (40 ng). The sediment is incubated for 20 minutes at 37° C, so that a bonding of the primer to the template-DNA can occur. The reaction sediment is

cooled down for 10 minutes at room temperature and then either immediately used for the labelling reaction or is frozen at -20°C. For the labelling reaction 3 µl labelling mixture, 1 µl [α-P^{32}]dATP and 2 µl T7-polymer (the T7-polymer was diluted 1:5 with enzyme dilution powder) are pipetted in the annealing reaction sediment and after briefly mixing are incubated for 5 minutes at room temperature. Meanwhile the already prepared sequencing mixture for the termination reaction (each 1 vessel with 2,5 µl 'G'-, 'A'-, 'T'- and 'C'-Mix "short") is preheated at 37° C. After completion of the labelling reaction 4 µl thereof is added to each of the four sequencing mixtures and briefly mixed by means of the pipette. The termination reactions are incubated for 5 minutes at 37° C. For the conclusion of the termination reaction 5 µl stop solution is added respectively. The sediments are now transferred into an incubator at 95° C, denaturized for 2 minutes and then placed on ice. Each 2,5 µl of the reactions are spread on a sequencing gel [25,2 g carbamide, 22 ml $H_2O_{bidest,}$ 6 ml 10x TBE, 10 ml polyacryl amide (40 %), 2 ml ammonium persulfate (16 mg/ml), 60 µl TEMED] in the sequence of a 'G', 'A', 'T', 'C'. The electrophoresis occurs at 40 Watt and 1 500 Volt for 4,5 hours.

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PATENT CLAIMS

1. Plasmid with a DNA sequence as represented in Figure 1 and their allel variations.

2. Plasmid with a DNA sequence as represented in Figure 4 and their allel variations.

3. DNA sequences with or without the nucleotide sequences as represented in Figure 1.

4. DNA sequences with or without the nucleotide sequences as represented in Figure 4.

5. Use of the plasmids or DNA sequences according to claims 1 to 4 in the microbiological analytics and/or diagnostics.

6. Use of the plasmids or DNA sequences according to claims 1 to 4 for medicinal and/or nutritional physiological or probiotical purposes.

7. Use of the plasmids or DNA sequences according to claim 1 to 4 as expression vectors.