Construction of Pseudomonas Aeruginosa PAK arcR Gene Knockout Mutant

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Abstract: New gene associated with Pseudomonas aeruginosa aminoglycoside antibiotic resistance was discovered by applying Mu transposon recombination technology, named arcR. In this experiment, partially deleted arcR gene and its upstream and downstream fragments were amplified from the genome of Pseudomonas aeruginosa PAOI, and taking suicide plasmid pEX18Tc as vector, to construct homologous recombination vector pEX-△arcR of arcR gene, and then the recombinant vector was transferred into Pseudomonas aeruginosa PAK by means of conjugal transfer. Then, single-exchange strains were screened out through antibiotic phenotypes and genotypes PCR detection, and double-exchange strains were screened out through SacB gene sucrose lethal effect. Compared with traditional electric conversion method, this method increases the conversion rate and reduces the false positive rate, and the correct rate of PCR positive detection reaches 100%, which solves the problem that some genes of Pseudomonas aeruginosa are difficult to knock out by electric conversion method. By sequencing analysis of screened double-exchange strains, arcR gene lacks 219 bases, and ArcR gene knockout strain of Pseudomonas aeruginosa was successfully constructed. MIC (minimum inhibitory concentration) test showed that the resistance of arcR gene deleted strains to streptomycin was higher than that of wild strains.

1. Introduction

Pseudomonas aeruginosa is widely distributed in nature, human skin, intestine and upper respiratory tract. Pseudomonas aeruginosa is an important conditional pathogen, as well as one of the common pathogens causing serious hospital-acquired infections. The bacteria also often cause diseases such as chronic bronchitis and cystic fibrosis secondary infection, and it is also one of the important causes of death in patients with severe burns, traumatic infections, cystic fibrosis and advanced cancer patients, seriously endangering human health and life. With the increasing use of clinical antibiotic drugs, the emergence of multi-drug resistant Pseudomonas aeruginosa has caused great troubles for effective anti-infective treatment in hospitals. Therefore, the study of its drug resistance mechanism is of great significance for the prevention and treatment of infections caused by Pseudomonas aeruginosa.

New gene associated with Pseudomonas aeruginosa aminoglycoside antibiotic resistance was discovered by applying Mu transposon recombination technology, named arcR. Compared with wild strain, streptomycin resistance of Mu mutant of arcR gene increases 16 times, while gentamicin resistance increased 4 times. Antibiotic resistance as a screening marker is a common method in research, however, the application of resistance markers is limited in the study of antibiotic resistance-related genes. Since PA0058 is an antibiotic resistance suppressor gene, antibiotic resistance box cannot be inserted in the construction of the knockout mutant, therefore, the method of direct absence is adopted, and single-exchange screening has also been improved from double-resistance screening to PCR identification. On the one hand, it improved the accuracy of screening, on the other hand, the problem of limited application of resistance markers was solved.

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2. Materials and Methods

2.1. Materials

2.1.1. Strains and Plasmids

Pseudomonas aeruginosa standard strain PAK, Clinical isolate strain PA68, mutant strain M122(PA68,arcR∷Mu), Suicide plasmid pEX18Tc.

2.1.2. Reagent

Tryptone and yeast extracts are products of the British company Oxoid; sucrose was purchased from Sigma, USA; agar powder and Pseudomonas isolated agar (PIA) were purchased from Difco, USA; agarose (Spanish packaging); tetracycline, streptomycin and ampicillin were purchased from Dingguo Biological Products Co., Ltd.; various restriction enzymes, T4 DNA ligase, Taq DNA polymerase, dNTP, DNA molecular weight standards DL2000, DL15000, IPTG, X-Gal are products of TaKaRa; all other reagents are domestic analytical purity.

2.1.3. PCR Primers

The primers used in this paper are shown in Table 1.

Table 1 Primer names and sequences.

Name	Sequence	Name	Sequence
arcRU1	CGGAATTCGGCAAGACCCTCAGCACTAT	arcRU2	GGGGTACCGGCCGAGTCGAATACCG
SacB1	ACGGAAGAATGATGTGCT	SacB2	TTTGCGAAAGAAACGAAC
arcRD1	GGGGTACCCGGCTGATGAACCGTCTCG	arcRD2	GCTCTAGACGGCAGTGATGGCGAAACC
Det1F	AGCAACTAACATGGCAAGCCTCTGA	Det1R	TGAAAATCTTCTCTCATCCGCCAAA
Det2F	AAGCAACTAACATGGCAAGCCT	Det2R	CTGAACAGCAAGGTGGCAAT
arcRrF	GCTCTAGAGATGAACGACCTCACCCTGC	arcRrR	CGGAATTCCACGGCAGTGATGGCGAAA

2.2. Construction of Recombinant Plasmid pEX-ΔarcR

Using Pseudomonas aeruginosa PAK genome as template, designed a pair of primers upstream and downstream of arcR gene: arcRU1/U2 and arcRD1/D2, 95℃ for 10min,95℃ for 1min,63℃ for 45s,72℃ for 1min, 30 cycles, amplified a 995bp arcRU fragment and a 606bp arcRD fragment at 72℃ for 10min, and then cloned them into plasmid pUC18 after enzyme digestion and purification, and separately obtained plasmids pUC18-arcRU and pUC18-arcRD. The above three plasmids were digested by enzymatic digestion of EcoR I and Kpn I, Kpn I and Xba I, EcoR I and Xba I, respectively. Cloned the obtained arcRU and arcRD fragments into the same pUC18 plasmid at the same time, and obtained pUC18-ΔarcR. There should be two bands after cleavage with EcoR I and Xba I, and clone the 1.6kb arcR fragment onto pEX18Tc vector. Positive transformants were screened, and recombinant plasmid pEX-ΔarcR was obtained by restriction enzyme digestion.

2.3. Transfer of Recombinant Plasmid pEX-ΔarcR into Pseudomonas aeruginosa PAK

Recombinant plasmid pEX-ΔarcR was extracted and electrically transfected into Escherichia coli S17, and obtained conjugal transfer donor bacteria pEX-arcR/S17. Donor bacteria were inoculated on 3 ml Tc10-containing LB medium, inoculated with Pseudomonas aeruginosa PAK as a recipient strain in 3 ml LB medium, overnight culture at 37°C. Inoculated by 1%, cultivated at 37 °C until: donor bacteria OD600=0.4, recipient strain OD600=0.8. Centrifuged 5ml donor bacteria solution and 1ml receptor bacterial solution and then mixed them. The above suspension was transferred to

the filter, and the filter was placed on an antibiotic-free plate overnight. Washed the filter with 1-2ml LB medium, and took 200ul to coat Tc100 plate, and single-exchange strain was screened after 48h incubation at 37 ℃.

2.4. Single Exchange Screening and Identification

Picked a single colony from the Tc100 plate, and inoculated it in Tc50 LB liquid medium. Using SacB1/B2 as primer, completed 30 cycles for 10min at 95°C, for 1min at 95°C, for 45s at 51°C, for 50s at 72°C; detected whether the recombinant plasmid pEX-ΔarcR is integrated into the PAK genome by bacterial PCR, 72°C 10min; using Det1F/R as primer, completed 30 cycles for 10min at 95°C, for 1min at 95°C, for 45s at 59°C, for 2min30s at 72°C; detected the correct insertion position of the recombinant plasmid by bacterial PCR, 72°C 10min.

2.5. Second Section

Picked a certain single commutator to inoculate 3 mL of antibiotic-free LB medium, cultivated it for 8 hours at 37°C. Diluted the medium 100 times and applied it on the PIA plate containing 5% sucrose, contrast with PIA plate without sucrose. Picked a single colony on a sucrose-containing PIA plate, overnight culture at 37 °C. Taking arcRrF/R as primer, completed 30 cycles for 10min at 95°C, for 1min at 95°C, for 45s at 63°C, for 1min10s at 72°C; detected double-exchanged genotypes by bacterial PCR, 72℃ 10min, to determine if the knocked out gene is partially missing; then taking Det2F/R as primer, utilized the PCR program Det for bacterial liquid PCR to detect whether the position of the double exchange insertion is correct. The resulting positive strain was named PAKΔarcR.

2.6. Detection of PAKΔarcR Antibiotic Resistance Phenotype

Used the microdilution method to determine streptomycin MIC of Pseudomonas aeruginosa PA68, M122, PAK and PAKΔarcR. Diluted the overnight liquid culture solution by 1:1000 and added 200μL to the first hole of a 96-well microtiter plate, and then added 100μL of bacteria solution to 2 to 10 holes. Streptomycin at a final concentration of 256 mg/L was added to the first hole, and then took 100μL into the second hole after mixing; diluted to the last hole in this way. Observed after incubation at 37 ° C for 20 hours, and then determined streptomycin MIC of PA68, M122, PAK and PAKΔarcR. PAK as positive control, observe whether the antibiotic resistance phenotype of the defective strain PAKΔarcR is consistent with the mutant strain M122, to detect whether the arcR gene in the model strain P. aeruginosa PAK has the same phenotype after inactivation.

3. Results

3.1. Construction of Recombinant Plasmid.

3.1.1. Subsection Titles

The arcRU fragment and arcRD fragment obtained by PCR were clonded onto the pUC18 vector, to obtain the recombinant plasmid pUC18-ΔarcR. The recombinant plasmid was digested with EcoRI and XbaI, 2.7 kb linearized carrier band and 1.6 kb foreign band should be obtained; digested with EcoRI, KpnI and XbaI, 2.7 kb linearized carrier band, 995bp exogenous arcRU fragment and 606bp exogenous arcRD fragment shoul be obtained. The agarose gel electrophoresis test result is shown in Figure 1. It can be seen from the figure that the electrophoresis results of PCR and plasmid digestion behavior are correct. To sequence this plasmid, the sequencing results indicated that the recombinant plasmid was successful.

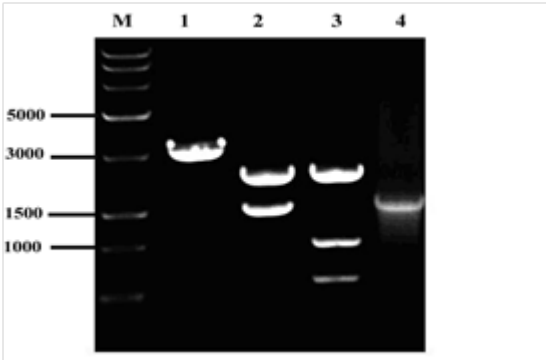

Figure 1 Determination of recombinant plasmid pUC18-ΔarcR.

(M:DNA molecular weight standard DL15000, 1: pUC18-ΔarcR plasmid EcoRI digestion product; 2: pUC18-ΔarcR *Eco*RI and *Xba*I digestion product; 3: pUC18-ΔarcR *Eco*RI, *Xba*I and *Kpn*I digestion product; 4: PCR product)

3.1.2. Construction of pEX-ΔarcR

The plasmid pUC18-ΔarcR was extracted, and there were two bands after digestion with EcoRI and XbaI. The 1.6 kb ΔarcR fragment was cloned into the pEX18Tc vector. Positive transformant was screened, for enzyme digestion detection. The electrophoresis result (shown in Figure 2) shows that the result is correct, and it indicates that the plasmid was successfully constructed, which be used for next step.

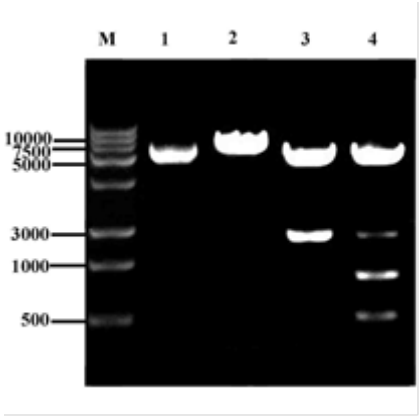

Figure 2 Enzyme digestion detection of plasmid pEX-ΔarcR.

(M:DNA molecular weight standard DL15000, 1: pEX-ΔarcR; 2: pEX-ΔarcR EcoRI digestion product; 3: pEX-ΔarcR EcoRI and XbaI digestion product; 4: pEX-ΔarcR EcoRI, XbaI and KpnI digestion product)

3.2. PAKΔarcR Screening and Identification.

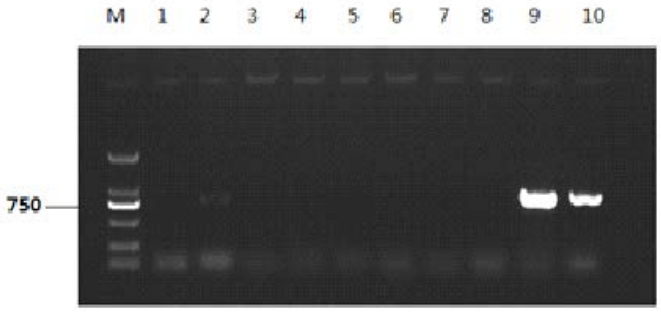

Figure 3 PCR amplification of sacB gene detection single exchange mutant.

(M: DL2000, 1-10: PCR amplification of sacB gene fragment)

In recombinant plasmid pEX-ΔarcR, 219 bases are missing in arcR gene, the upstream and downstream of the arcR fragment form two homologous arms to replace the homologous sequences in the PAK genome. Transform the recombinant plasmid into wild PAK competent cells, the arcR fragment carried on the plasmid will replace the normal arcR gene in the PAK genome, that is, the normal arcR gene in the PAK genome will be replaced and obtain defective strain PAKΔarcR. The detection of recombinant strains was divided into two steps: firstly, single-exchanged transformant was amplified by PCR to obtain 792bp sacB gene fragment and 2.1 kb Det1 fragment, the results shown in Figure 3 and Figure 4.

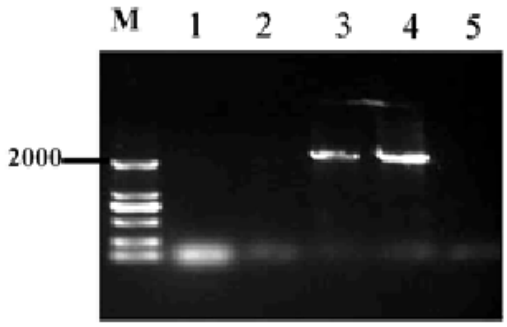

Figure 4 PCR amplification of Det1 fragment detection single exchange insertion site.

(M: DL2000, 1-5: PCR amplification of Det1 fragment)

Screened out the double-exchange transformers that can grow on sucrose-containing plates. Randomly selected some transformants to do colony PCR. Recombinant strains with double exchange could not amplify sacB gene fragments, but obtained the arcR gene fragment lacking 219bp. Recombinant detection primers arcRrF and arcRrR could amplify 888 BP arcRr fragments. In the absence of double exchange, PCR could amplify 1107bp fragment, the electrophoresis results shown in Figure 5. To verify the location of double exchange, amplified fragment Det2 with detection primers Det2F and Det2R, the electrophoresis results shown in Figure 6

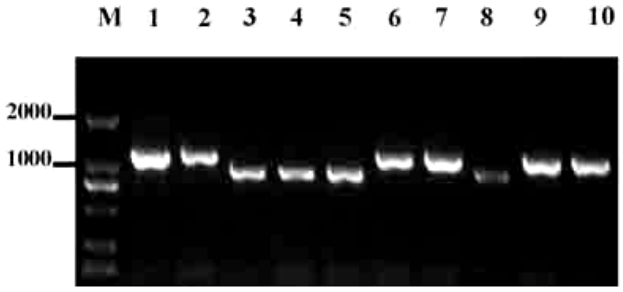

Figure 5 Detection of double-exchange by PCR amplification of arcRr fragments.

(M: DL2000, 1-10: PCR amplification of ArcRr Fragment)

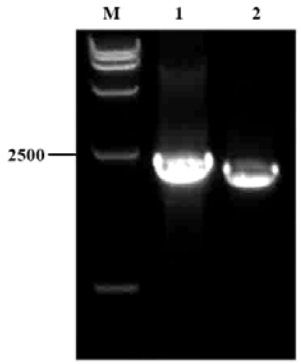

Figure 6 Detection of double Exchange Location by PCR Amplification of Det2 Fragment.

(M: DL2000, 1: negative control, complete arcR gene fragment; 2: ΔarcR fragment lacking 219bp)

3.3. PAKΔarcR Streptomycin MIC Detection.

Streptomycin MIC of Pseudomonas aeruginosa PA68, PAK, M122 and PAKΔarcR were respectively 16mg/L, 16mg/L, 256mg/L and 64mg/L. Compared with wild-type PAK, the MIC of knockout strain on streptomycin increased from 16mg/L to 64mg/L, while streptomycin MIC of M122(arcR∷Mu) was 256mg/L, 16 times higher than wild strain PA68. The phenotype of PAKΔarcR was consistent with the Mu mutant M122, and more resistant to streptomycin. It indicated that in Pseudomonas aeruginosa clinical isolate PA68 and standard strain PAK, inactivation of arcR gene affected streptomycin resistance of Pseudomonas aeruginosa.

4. Discussion

In this study, plasmid pEX18Tc was used as a homologous recombination vector for P. aeruginosa gene knockout. This plasmid is a suicide plasmid, containing tetracycline resistance gene and the sucrose-sensitive gene sacB. Single-transformed transformants that were screened by tetracycline alone were severely false positive, whereas the arcR gene is associated with antibiotic resistance, and resistance genes was not allowed to introduce for double and multiple resistance screening, so a pair of primers Det1F/R was designed based on the upstream sequence of the homologous recombination site and the partial sequence of the pEX18Tc vector backbone. The transformant with 2.1 KB band obtained by PCR amplification is positive. The data showed that the positive rate of single exchange was 25%; and the positive rate of PCR was 100%, avoiding 75% false positive. Double exchange transformant was screened with selective pressure by 5% sucrose concentration, and the probability of double exchange detected by PCR is 50%. The probability of transferring plasmids into PAK by conjugal transfer is higher than that of transferring plasmids into PAK by electroporation to obtain positive single exchange transformants. Although more single-exchange colonies could be obtained by electrotransformation of plasmids into PAK, but almost all colonies were false positive. In summary, using pEX18Tc as carrier, to utilize conjugal transfer method to construct Pseudomonas aeruginosa arcR gene knockout strain could solve the technical problem of high false positive rate of single exchange and no transformer can be obtained by electroporation, and avoid the effect of introducing resistance genes on the study of resistance genes

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