Efficient Conjugal Transfer of Inc R-plasmids at High Salt Concentration in the Mating Media

Fujio Hayashi,*1 Yasuhisa Araki,*2 Matsuhisa Inoue,*2 and Hajime Hashimoto*2
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There is an optimal salt concentration of mating in the conjugal transfer of an R plasmid, and incompatibility (Inc) group C R-plasmids are characteristic in that they demand high salt concentration such as 0.34 M or more in the efficient R-transfer. Both sodium and potassium salt are effective but divalent cation (Mg) are not.

Many pili-like structure of Escherichia coli carrying an Inc C plasmid Rms 417 were observed on an L agar plate which contained 0.34 M salt, but few in the absence of salt. Therefore, the pili could be considered sex pili that E. coli R+ produced in the presence of a moderate amount of monovalent salt.

We have previously analyzed on drug resistance and R plasmids in the drug resistant bacteria isolated from various fish and their environmental water.1–4) In the survey of R plasmids were isolated from Aeromonas hydrophila and Vibrio anguillarum, we found many experimental conditions which improved the efficiency of R isolation.5,4) The most important factor has been the usage of an adequate recipient organisms,6) and thus we have been able to isolate many R plasmids from A. hydrophila and V. anguillarum. These plasmids almost belong to incompatibility (Inc) group C.

R plasmids from V. anguillarum or other Vibrio species from marine fish are known to express low transferability among an Escherichia coli host.2,3,7) In the present study, transferability was clearly improved by increasing the salt concentration in a mixed culture.

Materials and Methods

Strains and R Plasmids Used

E. coli ML1410 F– met nal, E. coli J53 F– pro met, E. coli J53-1 F– pro met nal, and E. coli C rif were used as donors or recipients of R transfer. R plasmids used are listed in Table 1. Rms417 was derived from V. anguillarum5) and pJA4620 was initially thought to be nontransferable among E. coli (Dr. T. Arai, personal communication), but the transfer frequency from Vibrio sp. to E. coli was $10^{-4}$ and less than $10^{-8}$ from E. coli to Vibrio sp.5) The pJA4620 was derived from Vibrio sp. and was provided by Arai.8) Most of the other R plasmids were derived from an environmental source, or were provided by Dr. N. Datta (Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital) for the test of plasmid incompatibility.9)

Drugs and Media

Drugs used the concentration for selecting resistant bacteria were: tetracycline (TC, Lederle), 12.5 μg/ml; chloramphenicol (CM, Sankyo) 12.5 μg/ml; streptomycin (SM, Kyowa Hakko) 25 μg/ml; sulfanilamid (SA, Dainihon), 100 μg/ml; kanamycin (KM, Meiji), 25 μg/ml; ampicillin (APC, Toyama), 50 μg/ml; nalidixic acid (NA, Daiichi), 50 μg/ml; rifampin (RIF, Daiichi), 100 μg/ml. These drugs were purchased commercially. For cultivating bacteria, the following media were used: Antibiotic medium 3 (PA), Mueller-Hinton, Pepton broth, Brain heart infusion (Difco), Heart infusion (Nissui), Medium A.10) Sodium chloride was added to each medium in a final concentration of 0.0–0.7 M.

Transfer of R Plasmids

An overnight culture of donors and recipients were diluted 10 fold with fresh media and incubated

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Table 1. R plasmids used

<table>
<thead>
<tr>
<th>Host</th>
<th>R plasmid</th>
<th>Resistance marker</th>
<th>Incompatibility group</th>
<th>Original host</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli C</td>
<td>Rms417</td>
<td>TC. CM. SA</td>
<td>C</td>
<td>Vibrio anguillarum</td>
</tr>
<tr>
<td>E. coli J 53-1</td>
<td>Rhf 2</td>
<td>TC. SA</td>
<td>C</td>
<td>Aeromonas hydrophila</td>
</tr>
<tr>
<td>E. coli J 53-1</td>
<td>RA 1</td>
<td>TC. SA</td>
<td>C</td>
<td>Aeromonas hydrophila</td>
</tr>
<tr>
<td>E. coli J 53-1</td>
<td>R40a</td>
<td>SA. KM. APC</td>
<td>C</td>
<td>Salmonella typhimurium</td>
</tr>
<tr>
<td>E. coli C</td>
<td>pJA4620</td>
<td>TC. CM. SM. SA</td>
<td>E</td>
<td>marine Vibrio sp.</td>
</tr>
<tr>
<td>E. coli J 53</td>
<td>Rms151</td>
<td>TC. CM. SA. KM. APC</td>
<td>FII</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>E. coli ML1410</td>
<td>R144</td>
<td>TC. KM</td>
<td>Iα</td>
<td>Salmonella typhimurium</td>
</tr>
<tr>
<td>E. coli ML1410</td>
<td>R387</td>
<td>CM. SM</td>
<td>K</td>
<td>Shigella flexneri</td>
</tr>
<tr>
<td>E. coli ML1410</td>
<td>S-a</td>
<td>CM. SM. SA. KM</td>
<td>W</td>
<td>Shigella flexneri</td>
</tr>
<tr>
<td>E. coli ML1410</td>
<td>R391</td>
<td>KM</td>
<td>J</td>
<td>Proteus rettgeri</td>
</tr>
<tr>
<td>E. coli J 53-1</td>
<td>R446b</td>
<td>TC. SM</td>
<td>M</td>
<td>Proteus morganii</td>
</tr>
<tr>
<td>E. coli J 53</td>
<td>RP4</td>
<td>TC. KM. APC</td>
<td>P</td>
<td>Pseudomonas aeruginosa</td>
</tr>
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</table>

Table 2. Transfer frequency of various R plasmids in the mixed culture containing various concentration of NaCl

<table>
<thead>
<tr>
<th>Incompatibility group</th>
<th>Plasmids</th>
<th>Transfer frequency of R plasmid at various concentration of NaCl (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>Rhf 2</td>
<td>(&lt;10^{-8})</td>
</tr>
<tr>
<td>C</td>
<td>RA1</td>
<td>1.6 \times 10^{-8}</td>
</tr>
<tr>
<td>C</td>
<td>R40a</td>
<td>2.1 \times 10^{-9}</td>
</tr>
<tr>
<td>E</td>
<td>pJA4620</td>
<td>8.0 \times 10^{-8}</td>
</tr>
<tr>
<td>FII</td>
<td>Rms151</td>
<td>3.8 \times 10^{-4}</td>
</tr>
<tr>
<td>Iα</td>
<td>R144</td>
<td>1.3 \times 10^{-5}</td>
</tr>
<tr>
<td>K</td>
<td>R387</td>
<td>(&lt;10^{-8})</td>
</tr>
<tr>
<td>W</td>
<td>S-a</td>
<td>4.5 \times 10^{-8}</td>
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<tr>
<td>J</td>
<td>R391</td>
<td>3.8 \times 10^{-7}</td>
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<tr>
<td>M</td>
<td>R446b</td>
<td>5.8 \times 10^{-8}</td>
</tr>
<tr>
<td>P</td>
<td>RP4</td>
<td>4.0 \times 10^{-8}</td>
</tr>
</tbody>
</table>

* Donor, E. coli J53, J53-1, or ML1410; Recipient, E. coli C rif-r; Media, Antibiotic medium 3.

Fig. 1. Transfer of Rms417 (Inc C) and pJA4620 (Inc E) in two kind of broth under various concentration of NaCl (Rms417; ○-pepton broth, △-PA broth. pJA4620; ●-pepton broth, ▲-PA broth).
for about 2 h until the cells reached the late exponential phase (about 0.8 at OD 560). The cells were then washed 2 times with pepton broth by centrifugation and resuspended in a fresh medium to the same volume as before. The equal volumes of donor and recipient culture were mixed and incubated for 3 h at 37°C without agitation. The frequency of R transfer was calculated by dividing the number of transconjugant with the number of donor cells in the mixture.

Observation of Sex Pili

For scanning electron microscopy, cells were fixed for 3 h in cold 1.25% glutaraldehyde buffered at pH 7.4 with a 0.1 M cacodylate buffer containing 2% sucrose. The 20 µl cells, washed 2 times, were inoculated on a sterilized membrane filter (Nomura Micro Science Co. Ltd.).

The filters were placed on an L agar (10 g trypton, 5 g yeast extract, 1 g glucose, 5 g NaCl, 16 g agar per liter) plate containing with or without 0.34 M salt, respectively, and incubated for 3 h at 37°C. After this initial fixation, the cells were washed overnight with 2-3 changes of the same buffer containing 3% sucrose. The 20 µl cells, washed 2 times, were inoculated on a sterilized membrane filter (Nomura Micro Science Co. Ltd.).

The filters were placed on an L agar (10 g trypton, 5 g yeast extract, 1 g glucose, 5 g NaCl, 16 g agar per liter) plate containing with or without 0.34 M salt, respectively, and incubated for 3 h at 37°C. After this initial fixation, the cells were washed overnight with 2-3 changes of the same buffer containing 3% sucrose. Immersion for 1 h in a 0.1% solution of tannic acid in 0.1 M cacodylate buffer containing 3% sucrose and followed by the same buffer containing 3% sucrose and cacodylate buffered osmium tetroxide containing 1% sucrose. After dehydration in graded series of ethanol, the cells were transferred to isooamyl acetate, and dried in a critical point apparatus with liquid CO₂. The dried specimens were mounted on columnar and metal stubs, coated with platinum by ion sputtering and examined with a Hitachi S-700 scanning electron microscope.¹¹)

Results

NaCl Effect to Transfer Frequency of Various R Plasmids

Several R plasmids belonging to different incompatibility groups were examined for transferability under the increasing salt concentration in PA broth with mixed culture (Table 2). All of the Inc C, Inc J and Inc E plasmids showed the highest transfer frequency at a salt concentration of 0.17-0.51 M. Inc Iα plasmid transferred at equally high frequencies under a salt concentration of 0.17 M and 0.34 M. As for other R plasmids, optimal concentration of salt was 0.17 M or less, but RP4 (Inc P) was not affect of a salt. The transfer frequencies at different host strains were equally in these experiments.

Effect of NaCl Concentration on the R Transfer

The transfer frequency of Rms417 or pJA4620 in E. coli was compared at different salt concentration of pepton broth or PA broth used for mixed culture (Fig. 1). Both R plasmids showed the highest transfer frequency at 0.25-0.34 M of salt in pepton broth or 0.17-0.51 M salt in PA broth. The transfer frequency was markedly decreased in the lower or the higher concentrations of salt. At a salt concentration of 0.08 M that is used most frequently for cultivation of strains of various species, Rms417 or pJA4620 transferred at far less efficiencies. R transfer was 10 times more efficient in PA broth than in pepton broth.
As we found distinct difference in the transfer frequency of R plasmids in PA and pepton broth, we further examined other media and the results are shown in Table 3. Antibiotic medium 3 was the best media for R transfer, and medium A was the worst. There was about 1000 times difference in the frequency of R transfer between those two. The effect of salt addition into various media was not so marked as in PA 3.

Specificity of Salt Affect

The affect of different salt on R plasmid transfer was then examined. As shown in Table 4, KCl was also effective in increasing R transfer frequency while the concentration of MgSO₄ did not effect on the frequency.

Transfer Frequency of R Plasmids in Various Media

As we found distinct difference in the transfer frequency of R plasmids in PA and pepton broth, we further examined other media and the results are shown in Table 3. Antibiotic medium 3 was the best media for R transfer, and medium A was the worst. There was about 1000 times difference in the frequency of R transfer between those two. The effect of salt addition into various media was not so marked as in PA 3.

Observation of Sex Pili

The formation of pili and flagella of E. coli X1038 was not observed during the logarithmic and stationary growth phase, but cells on the L agar plate without salt became elongated. On the other hand, many pili of E. coli carrying Rms417 were observed on L agar plate which contained 0.34 M salt, but very few without salt (Fig. 2).

Discussion

The above results indicate that there is an optimal concentration of mating media in the effective conjugal transfer of an R plasmid, and incompatibility group C R-plasmids are characteristic in that they demand a high salt concentration such as 0.34 M or more in the efficient R-transmission. Both sodium and potassium chloride are effective but a divalent cation (Mg) did not affect the transfer frequency. These charges would affect the isolation frequency of transferable Inc C plasmids in nature.

The salt concentration is considered to affect several steps in the mating process.

If salt affect is mediated through better piliation of the donor, or competency of the recipient, the addition of salt during the stage of preculture
should also increase the transfer frequency of R plasmids. However, the addition of salt during, the mixed culture was found to cause a marked increase in the transfer frequency of R plasmid. The degree of donor cell piliation is of no relevance since almost no pili will survive the washing before initiating the 3 h mating and the cells will rebuild them at the moderate concentration of monovalent salt. In view of the present results, salt concentration might affect the capture of recipient cells by donor pili.

Difficulty of R transfer was mostly observed in the case of interspecific conjugation. Most R plasmids are transmissible among Enterobacteriaceae but not to Pseudomonas or Vibrio. Inc P or Inc C plasmids are known as broad-spectrum plasmids and are transferable between different families. The factor responsible for such a broad specificity of R transfer was not analysed. Inc C R-plasmids have been found to transfer at higher frequencies even in the original host when the salt concentration was 0.34 M.*. Growth of cells and the R transfer would require independent functions and the necessity of higher salt concentrations in both phenotypes would reflect as similar evolutionary steps.

This suggests that Inc C group plasmids may increase in number if the R+ cells spread naturally from the fish-breeding pond to sea water and R plasmids would be transferred to cell to cell contact in the presence of a salt. This may present an important problem of drug resistance in marine fish pathogens but also public health to be solved.

Acknowledgement

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References
