Deletion Plasmids from Transformants of *Pseudomonas aeruginosa trp* Cells with the RSF1010-*trp* Hybrid Plasmid and High Levels of Enzyme Activity from the Gene on the Plasmid

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A RSF1010-*trp* hybrid plasmid which contained the tryptophan operon of *Escherichia coli* was introduced into *Pseudomonas aeruginosa trp* cells by transformation. From the Trp* transformants several deletion plasmids were obtained, and their physical maps with restriction endonucleases were constructed. *P. aeruginosa trp* cells with these plasmids showed at first more than 100 times higher levels of tryptophan synthetase β activity over that of the control *P. aeruginosa* wild-type cells, but these levels were drastically decreased by 1 week of successive transfers of cultures. This decrease in enzyme activity was found to be due to the change on the plasmids but not to the host cells. The production of *E. coli* tryptophan synthetase β enzyme in *P. aeruginosa* cells was proved by immunological test.

Four hybrid plasmids carrying the tryptophan operon of *Escherichia coli* were constructed in vitro and cloned in *E. coli* cells (13). One of the hybrid plasmids, RP4-*trp*, was conjugatively transferred to *Pseudomonas aeruginosa trp* cells, causing the constitutive synthesis of anthranilate synthetase (ASase) and tryptophan synthetase β (TSaseβ) enzymes (12). We have previously shown that RSF1010, which has a mass of 5.5 megadaltons (5.5 Mdal) and which confers resistance to streptomycin and sulphonamide (3), was potentially useful as a cloning vector also in *Pseudomonas putida* and *P. aeruginosa* cells (11). In this report, I describe the introduction of the RSF1010-*trp* hybrid plasmid, which has an *E. coli* tryptophan operon, into *P. aeruginosa trp* cell by transformation, yielding several deletion plasmids which have lost parts of the original RSF1010-*trp* hybrid plasmid.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* C600 r**-** m**-** trpB and *E. coli* C600 r**-** m**-** trpB containing RSF1010-*trp* strains were described (13). *P. aeruginosa* M12, which has a mutated site in *trpC* or *trpD*, was used as a recipient cell (9).

**Media.** Vogel and Bonner medium (18) and glutamate medium (9) were used for the minimal media of *E. coli* and *P. aeruginosa*, respectively. Glutamate minimal medium contained (in grams per liter): sodium glutamate, 20; glucose, 5; Na₂HPO₄, 12H₂O, 5.63; KH₂PO₄, 0.25; MgSO₄·7H₂O, 1.0.

**Transformation.** Transformation of *E. coli* (17) and *P. aeruginosa* (15) cells was as previously described. After transformations were carried out, the samples were immediately spread on minimal medium plates.

**Preparation of cleared lysates and isolation of plasmid DNAs.** Plasmid DNA from *E. coli* cells was purified according to the method of Tanaka and Weisblum (17). That from *P. aeruginosa* cells was purified by the method previously described (11) and stored in 0.1X SSC (15 mM NaCl and 1.5 mM sodium citrate) plus 1 mM ethylenediaminetetraacetic acid buffer (pH 7.5).

**Restriction endonucleases.** EcoRI and BamNI (identical with *BamHI*) restriction endonucleases were prepared according to the method of Tanaka and Weisblum (17) and Shibata and Ando (16), respectively. HindIII was purchased from Biolab and PstI from Boehringer Mannheim GmbH. Conditions for each endonuclease digestion were as follows. EcoRI: 10 mM MgCl₂, 50 mM NaCl, 100 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5); BamNI: 5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid, 50 mM Tris (pH 7.5); HindIII: 7 mM MgCl₂, 60 mM NaCl, 7 mM Tris (pH 7.4); PstI: 10 mM MgSO₄, 90 mM Tris (pH 7.5). Double digestion with EcoRI and HindIII was carried out in the buffer for HindIII, with EcoRI and PstI in the buffer for EcoRI. The digestion mixture was usually incubated at 37°C for 40 min in a total volume of 30 μl. The reaction was terminated by addition of 10 μl of BJ solution (17), and the mixture was heated at 65°C for 5 min before agarose gel electrophoresis.

**Agarose gel electrophoresis.** Electrophoresis in 0.7% agarose gels in glass tubes with an internal diameter of 0.5 cm and a height of 9 cm was run for 3 h.
RESULTS

Transformation experiment. P. aeruginosa M12 cells were transformed to Trp+ with RSF1010-trp hybrid plasmid DNA purified from E. coli C600 rbs−. About 100 transformants were obtained with 1 μg of RSF1010-trp DNA. All the transformants selected for tryptophan independence so far tested were resistant to 1 mg of streptomycin per ml on the plate. Five transformants were grown in 100 ml of liquid cultures, and the plasmids were purified and used for further study.

Structure of each plasmid. Only one (RSF1010-trpΔO) of five plasmids purified from the transformants was similar to the original RSF1010-trp in the digestion pattern with EcoRI (data not shown), which yielded 10.8- and 5.5-Mdal fragments by two sites susceptible to EcoRI on the plasmid. The 10.8-Mdal fragment contained the E. coli tryptophan operon, and the 5.5-Mdal fragment was a linear form of the RSF1010 plasmid (13). The four other plasmids were cut at only one site with EcoRI, yielding one fragment less than 16.3 Mdal in size (data not shown). These were designated RSF1010-trpΔ1, RSF1010-trpΔ2, RSF1010-trpΔ3, and RSF1010Δ1, in decreasing order of molecular weights. To determine which part of the original RSF1010-trp plasmid DNA was deleted in each deletion plasmid, double-digestion experiments were done as follows. Double digestion of RSF1010-trp and RSF1010-trpΔO with EcoRI and HindIII yielded seven fragments corresponding to 5.5, 3.0, 2.8, 1.9, 1.8, 1.1, and 0.2 Mdal (Fig. 1a and f) (the 0.2-Mdal fragment can not be seen in this photograph). RSF1010 has no site susceptible to HindIII (data not shown). On the other hand, double digestion of RSF1010-trpΔ1 with EcoRI and HindIII yielded five fragments corresponding to 5.6, 2.8, 1.8, 1.1, and 0.2 Mdal (Fig. 1e); RSF1010-trpΔ2 yielded four fragments of 6.8, 2.8, 1.1, and 0.2 Mdal (Fig. 1d); RSF1010-trpΔ3 yielded four fragments of 5.4, 3.2, 1.1, and 0.2 Mdal (Fig. 1c); and RSF1010Δ1 yielded only one fragment of 4.8 Mdal (Fig. 1b).

Double digestion of the original RSF1010-trp and RSF1010-trpΔO with EcoRI and PstI yielded six fragments corresponding to 7.8, 5.0, 1.3, 0.93, 0.77, and 0.50 Mdal (Fig. 2a and f) (the 0.50-Mdal fragment cannot be seen in this photograph); RSF1010-trpΔ1 yielded 5.2, 5.0, and 1.3 Mdal (Fig. 2e) (fragments of 5.2 and 5.0 Mdal are overlapping each other in the gel); RSF1010-trpΔ2 yielded three fragments of 5.0, 4.6, and 1.3 Mdal (Fig. 2d); RSF1010-trpΔ3 yielded three
fragments of 5.0, 3.6, and 1.3 Mdal (Fig. 2c); and RSF1010Δ1 yielded only one fragment of 4.8 Mdal (Fig. 2b). As described above, all the plasmids except RSF1010Δ1 yielded the two fragments of 5.0 and 1.3 Mdal. RSF1010Δ1 cannot be digested with HindIII or PstI alone, and it showed a digestion pattern by HincII similar to that of RSF1010 plasmid itself (data not shown). RSF1010 plasmid has two sites susceptible to PstI, one of which is located very close to the EcoRI site and the other 0.5 Mdal away from the EcoRI site (unpublished data). RSF1010-trp and RSF1010-trpΔ0 have a site susceptible to BamNI in the 10.8-Mdal EcoRI fragment which is located 0.8 Mdal away from the EcoRI site, but other four deletion plasmids could not be digested with this enzyme (data not shown). From these results and the mapping of λtrp phage DNA (7), the physical maps of the five plasmids with restriction endonucleases were determined as shown in Fig. 3.

Transforming ability of each plasmid. Table 1 shows the transforming ability of each plasmid for P. aeruginosa M12 and E. coli C600 rts− mts− trpB cells. Each plasmid had a relatively high transforming ability for both bacteria, indicating that each plasmid has at least trpB and trpC or trpD genes. From other experiments, it was verified that the DNA preparation of RSF1010Δ1 contained also a small amount of the RSF1010-trpΔ0 plasmid, and that RSF1010Δ1 alone has no transforming activity for tryptophan independence but it does have activity for streptomycin resistance (data not shown).

ASase and TSaseβ activities. The crude extracts of the cells containing each plasmid except RSF1010Δ1 and RSF1010-trpΔ3 showed levels of TSaseβ activity more than 100 times higher than that of the control P. aeruginosa PA01 cells (Table 2). ASase activities from the cells with plasmids were also high compared with that of the control, but the values were only two to three times higher than that of the wild cells (data not shown). To know whether or not high levels of TSaseβ activity can be maintained during culture, cells with plasmids were cultured for a week by successive daily transfers of culture. After 1 week, the TSaseβ activities in the cells were measured. TSaseβ activities in the cells containing RSF1010-trpΔ0, Δ1, and Δ2 were decreased drastically in TSaseβ (Table 2), whereas ASase activity remained in the same level (data not shown).

From the map in Fig. 3 and the low activity of TSaseβ enzyme (trpB gene product) shown by RSF1010-trpΔ3—harboring cells, it may be considered that the RSF1010-trpΔ3 plasmid has lost an effective promoter or a part of a functional trpB gene.

How did the decrease of TSaseβ activity occur? To explore whether the decrease in TSaseβ enzyme activity was caused by a change in the host cells or the plasmid itself, P. aeruginosa M12 cells were transformed to tryptophan independence with RSF1010-trpΔ0 plasmid DNAs purified from the cells before and after 1 week of culture. Then each pair of transformants were cultured in 100 ml of glutamate minimal medium, and the specific activity of TSaseβ enzyme in the cells was measured. Two transformants, having RSF1010-trpΔ0 purified from the cells before 1 week of culture, showed values of 209 and 104; on the other hand, two transformants having RSF1010-trpΔ0 purified from the cells after 1 week of daily subculture showed values of 27.8 and 3.3. It may be concluded that the decrease in TSaseβ activity by 1 week of culture was due to some change of the plasmid rather than to that of the host cells. Each of the four plasmids purified from the cells after 1 week of daily subculture showed digestion patterns by EcoRI and HindIII on agarose gel electrophoresis identical to those from the cells before 1 week of culture (data not shown), and therefore additional deletions are not responsible for the decrease in TSaseβ activity.

**Fig. 2. Double digestion pattern of each plasmid DNA with EcoRI and PstI.** (a) RSF1010-trp, (b) RSF1010Δ1, (c) RSF1010-trpΔ3, (d) RSF1010-trpΔ2, (e) RSF1010-trpΔ1, (f) RSF1010-trpΔ0. (g) phage λch877 DNA with HindIII.
Immunological tests for *E. coli* TSaseβ enzyme synthesis in *P. aeruginosa* cells. Ouchterlonpy experiments were employed to determine whether the TSaseβ enzyme in *P. aeruginosa* cells containing RSF1010-trpΔ0 is identical to that in *E. coli*. Figure 4 shows that extracts from *E. coli* cells containing RSF1010-trp and *P. aeruginosa* cells containing RSF1010-trpΔ0 reacted with the antiserum to the *E. coli* TSaseβ enzyme, giving distinct precipitation lines which fused completely with each other. This indicates that *E. coli* TSaseβ enzyme was synthesized from RSF1010-trpΔ0 plasmid in *P. aeruginosa* cells.

**DISCUSSION**

The mechanisms of yielding deletion plasmids in this report are not clarified yet, but some experimental results were obtained with respect to this phenomenon. It was sometimes observed that even if the original plasmid was obtained from the cells of the transformant at first, deletion plasmids were obtained from the cells of the same transformant in subsequent experiments. Once a deletion plasmid was formed, further deletion did not occur. Deletion plasmids were also obtained from the transformants of the cells which were heated at 42°C to inactivate restriction systems of the *P. aeruginosa* host cells (6) for more than five generations before the trans-
formation. This suggests that deletion plasmids were not created by some deoxyribonuclease-like restriction endonuclease. I also introduced RSF1010-trp plasmid into P. putida cells by transformation (unpublished data). In this case, so far tested, no deletion plasmids could be obtained. From these observations, it seems to be most conceivable that a part of the original RSF1010-trp plasmid DNA unnecessary to P. aeruginosa M12 cells was cut off by some mechanism(s) and that the deletion plasmids conferred on the cell some advantages over the original plasmid for growth.

In spite of selecting transformants only for tryptophan independence on the minimal plate at first, RSF1010Δ1 plasmid was obtained. This plasmid was proved to have no transforming ability for tryptophan independence but to have ability for streptomycin resistance. It was also verified that the cells carrying RSF1010Δ1 have also RSF1010-trpΔ0 in a small amount. Since P. aeruginosa cells were cultured under conditions for selecting streptomycin (100 μg/ml) resistance and tryptophan independence for the purification of the deletion plasmids, RSF1010Δ1 is supposed to have an advantage over the RSF1010-trpΔ0 with respect to streptomycin resistance.

From the physical map of each deletion plasmid with restriction endonucleases, RSF1010-trpΔ1, RSF1010-trpΔ2, and RSF1010Δ1 seemed to be formed by deleting a part of the original RSF1010-trp plasmid. On the other hand, RSF1010-trpΔ3 seemed to be formed by deleting at least two parts of the original RSF1010-trp plasmid.

The high levels of TSaseβ enzyme activity in the cells carrying RSF1010-trpΔ0, -Δ1, and -Δ2 must have conferred disadvantage with respect to the growth of P. aeruginosa cells. That cells having a lower level of TSaseβ enzyme activity were selected during 1 week of daily subculture may be the reason for the drastic decrease of TSaseβ enzyme activity.

On the other hand, ASase activities were not as high as TSaseβ enzyme, and no distinguishable decrease in ASase activity was observed after 1 week of culture. Both TSaseβ and ASase activities were not repressed by the exogeneous tryptophan (data not shown), as in the case of the RP4-trp hybrid plasmid in P. aeruginosa cells (12).

The amounts of the plasmid DNAs from the cells after 1 week of daily subculture seemed to be similar to those from the cells before 1 week of culture, and the restriction endonuclease digestion patterns of the plasmids were identical.

I suggest that high levels of TSaseβ activity in P. aeruginosa cells containing RSF1010-trp derivatives were caused by constitutive transcription from the promoter, which was masked at low level in E. coli cells, and that the drastic decrease in TSaseβ activity was caused by some structural change of the promoter region. At present which promoter, among P+, P+, P+, that of RSF1010 plasmid, and the trp internal promoter at the operator distal end of the trpD gene in E. coli tryptophan operon, takes part in this phenomenon is not clear.

In immunological experiments, a crude extract of the control P. aeruginosa wild-type cells gave no precipitation line with the antiserum to the TSaseβ enzyme of E. coli, probably because of the low level of enzyme in the crude extract. Hedges et al. (4) reported that TSaseβ of P. aeruginosa was neutralized effectively by antiserum prepared to the TSaseβ of P. putida but poorly by antiserum to the TSaseβ of E. coli. This agrees with the result of the Ouchterlony experiment in this report, which shows the production of E. coli TSaseβ in P. aeruginosa cells.

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LITERATURE CITED