Integration of Replication-defective R68.45-like Plasmids into the *Pseudomonas aeruginosa* Chromosome

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R68.45 and other similar broad-host-range (IncP) plasmids carrying a tandem repeat of the 2.1 kb insertion element IS21 mobilize the chromosome of many different Gram-negative bacteria. To analyse the structure of R68.45–chromosome cointegrates, whose involvement in the mobilization process had been postulated previously, we selected for the stable integration of R68.45-like plasmids into the *Pseudomonas aeruginosa* chromosome. Two plasmids were chosen: pME28, a transfer-deficient, mobilizable RP1 derivative with an inactive replication control (*trfA*) gene, and pME487, an R68.45 derivative with a *trfA*(ts) mutation causing temperature-sensitive replication. Chromosomally integrated pME28 and pME487 were found to be flanked by single IS21 elements. This structure is in agreement with a ‘cut-and-paste’ mode of R68.45 transposition. pME28 and pME487 showed a low specificity of insertion but rarely (<0.1%) induced auxotrophic mutations. Hfr (high-frequency-of-recombination) donors of *P. aeruginosa* could be obtained by chromosomal integration of pME487 or pME28; in the latter case, the transfer functions lacking from pME28 had to be provided *in trans* on an autonomous plasmid. Hfr donors gave higher conjugational linkage and transferred longer stretches of the *P. aeruginosa* chromosome than did R68.45 donors. This suggests that the integration of R68.45 into the donor chromosome is short-lived in *P. aeruginosa*.

INTRODUCTION

The broad-host-range IncP plasmid R68.45 mobilizes the chromosome of a large number of Gram-negative bacterial species (Holloway, 1979; Haas & Reimmann, 1988). The 2.1 kb insertion element IS21, which occurs as a single copy on the parental plasmid R68 ($\cong$ RP1), is tandemly repeated on R68.45 (Riess *et al.*, 1980; Willetts *et al.*, 1981). The IS21 tandem repeat is essential for the chromosome-mobilizing ability (Cma) of R68.45 (Currier & Morgan, 1982; Haas & Riess, 1983). In *Pseudomonas aeruginosa*, R68.45 mobilizes the chromosome from many origins and in both orientations (Haas & Holloway, 1978). It has been postulated that Cma results from the random integration of R68.45 into the chromosome via IS21-dependent transposition (Willetts *et al.*, 1981). However, stable chromosomal integration of R68.45 has not been observed; that is, spontaneous Hfr (high-frequency-of-recombination) strains have not been isolated from R68.45 donors in different bacterial species (Haas & Reimmann, 1988). Hence the structure of the postulated R68.45–chromosome cointegrate is unknown. It appears that replication-proficient IncP plasmids are not maintained stably in the chromosome of *Escherichia coli* or *P. aeruginosa* without specific selective pressure (Martin *et al.*, 1981; Grinter, 1984; Reimmann & Haas, 1986).

When R68.45 mobilizes nonconjugative plasmids in *E. coli*, cointegrates are formed which are stable in *recA* strains. Single IS21 copies in the same orientation are found at the junctions between R68.45 and the mobilized plasmid (Riess *et al.*, 1983). Current evidence suggests that

Abbreviations: Cma, chromosome-mobilizing ability; ts, temperature-sensitive.

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Table 1. *Bacterial strains and plasmids*

<i>E. coli</i>	Genotype/phenotype*	Reference or construction
S17-1	<i>pro thi hsdR hsdM⁺ recA</i> ; chromosomally integrated RP4-2 (Tn1::ISR1, Tc::Mu, Km::Tn7)	Simon <i>et al.</i> (1983)
ED8654	<i>metB hsdR hsdM⁺ supE supF</i>	Borck <i>et al.</i> (1976)
<i>P. aeruginosa</i>		
PAO236	<i>ilv-226 hisI14 lysA12 met-28 trp-6 proA82 nalA2</i>	Haas & Holloway (1976)
PAO6049	<i>met-9011 amiE200 strA</i>	Rella <i>et al.</i> (1985)
PAO6231	<i>ilv-226 hisI14 lysA12 ser-3 trp-6 proA82</i>	This work†
PAO6236	<i>met-9011 amiE200 strA trpE::pME28‡</i>	This work
PAO6049 <i>ura::pME28</i>	<i>met-9011 amiE200 strA ura::pME28</i>	This work
PAO6049::pME28-5,	<i>met-9011 amiE200 strA</i> ; chromosomally integrated pME28	This work
PA06049::pME28-9,		
PA06049::pME28-17 }		
PTO6017	<i>leu-10 rec-102 strA</i> ; chromosomally integrated pME14 (=pME134::IS21)	Reimmann & Haas (1986)
PTO6025	<i>leu-10 rec-102 strA</i>	Reimmann & Haas (1986)
PTO6034	<i>leu-10 rec-102 strA</i> ; chromosomally integrated pME487	This work (Fig. 4)
PTO6038, } PTO6039, } PTO6040 }	<i>leu-10 rec-102 strA</i> ; chromosomally integrated pME487	This work
Plasmids		
R68.45	Ap/Cb Tc Km Tra Cma IncP-1; (IS21) ₂	Haas & Holloway (1976)
pACYC184::IS21-91	Tc Cm P15A replicon	Riess <i>et al.</i> (1983)
pME28	Tc Km Δ(Tra2-Tra3-TrfA) IncP-1; (IS21) ₂	Reimmann & Haas (1987); Fig. 1(b)
pME206	Ap/Cb Km Tra IncP-1	Rella <i>et al.</i> (1987)
pME301	Ap/Cb Tc Km Tra Rep(ts) IncP-1	Watson (1980)
pME472	Tc Km Tra Cma IncP-1; (IS21) ₂	This work (Methods)
pME487	Tc Km Tra Cma Rep(ts) IncP-1; (IS21) ₂	This work (Fig. 1a)
pUB307	Tc Km Tra IncP-1	Bennett <i>et al.</i> (1977)

*Symbols are according to Holloway & Morgan (1986) and Novick *et al.* (1976). Tandem repeats of IS21 are denoted by (IS21)₂.

†*met-28* of PAO222 (Haas & Holloway, 1976) was replaced by *ser-3* of PAO2 (Isaac & Holloway, 1968).

‡Growth of PAO6236 with an anthranilate supplement indicates mutation in *trpE*.

IS21 transposase preferentially cuts R68.45 between the tandemly repeated IS21 copies and then the entire R68.45 plasmid is inserted into the target replicon (Reimmann & Haas, 1987). This model leads to two predictions concerning the integration of R68.45-like plasmids into the bacterial chromosome. (i) The integrated plasmid should be flanked by single IS21 copies. (ii) Integration should produce chromosomal mutations. As we show here, both predictions hold true when replication-defective IncP plasmids carrying an IS21 tandem repeat are integrated into the *P. aeruginosa* chromosome. From linkage data we infer that cointegrates between the replication-proficient R68.45 and the *P. aeruginosa* chromosome are unstable.

METHODS

Strains and plasmids. These are listed in Table 1.

Media, growth conditions, and genetic techniques. They have all been described before (Stanisich & Holloway, 1972; Haas & Holloway, 1976; Reimmann & Haas, 1986, 1987).

Carbenicillin enrichment for tetracycline-sensitive cells. Overnight cultures of *P. aeruginosa* PAO6049::pME28 were diluted into fresh nutrient broth and grown with shaking to 10⁸ cells ml⁻¹. Tetracycline was added to a final concentration of 20 µg ml⁻¹. After 1 h incubation, carbenicillin (final concentration 2 mg ml⁻¹) was added and incubation was continued overnight. Cells were washed and the enrichment cycle was repeated once. After plating on nutrient agar, tetracycline-sensitive derivatives, which had lost the integrated pME28, were identified by replica plating.

Fig. 1. Restriction maps of IncP plasmids with an IS21 tandem repeat. Heavy arrows denote IS21. Tc^R, tetracycline resistance; Km^R, kanamycin resistance; Tra1, Tra2, Tra3, transfer gene regions; oriV, origin of vegetative replication; oriT, origin of transfer; trfA(ts), replication control locus; ClaI*, restriction site protected by methylation in *dam*⁺ *E. coli* but not in *P. aeruginosa* (Haas & Riess, 1983). pME487 (a) is an R68.45 derivative constructed as described in Methods. pME28 (b) is an RP1 derivative (Reimmann & Haas, 1987). Note that R68 and RP1 are very similar plasmids (Willets *et al.*, 1981). The IS21 elements of pME28 are functionally indistinguishable from those of R68.45 (Reimmann & Haas, 1987).

DNA manipulations. Conditions for plasmid isolation, restriction, ligation and Southern transfer have been given elsewhere (Itoh *et al.*, 1984; Reimmann & Haas, 1986).

Construction of pME487. The 49 kb *EcoRI*–*BglII* fragment of R68.45 (Haas & Riess, 1983) and the 7.5 kb *EcoRI*–*BglII* fragment of pUB307 (=RP1ΔTn1; Bennett *et al.*, 1977) were ligated to produce pME472 (=R68.45ΔTn1). The 20 kb *SalI* fragment of pME472 carrying *trfA*⁺ was then replaced by the homologous 20 kb *SalI* fragment of pME301 [=RP1Rep(ts)], which carries a *trfA*(ts) mutation (Rella *et al.*, 1987). The resulting recombinant, pME487 (Fig. 1a), was temperature sensitive for replication at 43 °C in *E. coli* and in *P. aeruginosa* and self-transmissible, although transfer frequencies appeared to be lower than those of RP1. Restriction analysis of pME487 showed that a spontaneous deletion of about 0.1 kb had occurred in the 1.3 kb *BglII*–*SalI* fragment near *oriV*, but this had no apparent effect on the plasmid phenotype.

RESULTS

Chromosomal integration of pME487

We first chose pME487, a temperature-sensitive derivative of R68.45 without the TEM-2 β-lactamase transposon Tn1 (Fig. 1a), to study the interaction of R68.45-like plasmids with the *P. aeruginosa* chromosome. The temperature sensitivity of pME487 is due to mutation in the *trans*-acting replication control gene *trfA*. In *E. coli* pME487 formed cointegrates with pBR325 at frequencies of 10^{−3}–10^{−4}. These frequencies were calculated as the ratio of cointegrate transfer per pME487 transfer (i.e. transfer of chloramphenicol resistance per transfer of kanamycin resistance) in a mating-out assay with a streptomycin-resistant *E. coli* recipient (Reimmann & Haas, 1987). pME487 and pBR325 were joined together by single IS21 copies (data not shown), as expected from previous work on R68.45 (Riess *et al.*, 1983). pME487 was transferred from *E. coli* ED8654 to the recombination-deficient *P. aeruginosa* mutant PTO6025 at permissive temperature (30 °C); selection was made on nutrient agar containing streptomycin (3000 μg ml^{−1}) + kanamycin (200 μg ml^{−1}) + tetracycline (100 μg ml^{−1}). Overnight cultures of *P. aeruginosa* transconjugants were then plated onto the same medium at non-permissive

Fig. 2. Structure of chromosomally integrated pME487. (a) Southern hybridization of *Cla*I-digested chromosomal DNA with 35 S-labelled pACYC184::IS2I-9I as the probe. The chromosome is indicated by wavy lines. Abbreviations are the same as in Fig. 1. Lane 1, strain PTO6034; lane 2, pME487 control; lanes 3–5, strains PTO6038, PTO6039, and PTO6040, respectively. Bands D, E, F, G, H, I, M, O are chromosome-IS2I border fragments; band K is typical of an IS2I tandem repeat (found only in pME487); bands A, B, C, L, N are pME487-internal fragments; band A (~55 kb) occurred only in the pME487 control; band L (2.0 kb) is missing from the pME487 control because of methylation of the *Cla*I* site in *E. coli*. (b) Proposed structure of pME487 integrated into the *P. aeruginosa* chromosome. Letters refer to fragments in Fig. 2a.

temperature (43 °C). Resistant colonies appeared at a frequency of about 10^{-6} . They were purified several times until the kanamycin- and tetracycline-resistance markers were stably maintained at 30 °C and 43 °C. Some strains, which carried pME487 integrated in the chromosome, were analysed further (PTO6034, PTO6038, PTO6039, PTO6040). No autonomous plasmid could be detected in cleared lysates of these strains. The strains were Hfr donors, transferring proximal markers at about 10^{-3} per donor. The origin of strain PTO6034 was mapped between *proA* and *trp-6* (cf. Fig. 4). The other strains efficiently mobilized markers in the 0–20 min region of the chromosome but their origins were not mapped.

The structure of the integrated pME487 was analysed by Southern hybridization of *Cla*I-cut chromosomal DNA extracted from the four Hfr strains, with a 35 S-labelled IS21 probe (Fig. 2a). The 2.1 kb band which is typical of the IS21 tandem repeat in pME487 (Fig. 2a, lane 2) was missing in all cases. If single IS21 copies flank the integrated plasmid, two pME487-internal bands of 2.0 and 1.3 kb plus two border fragments of varying length are expected and this pattern was found (Fig. 2a, lanes 1, 3, 4, 5). In addition, two fragments of constant length (20 kb and 35 kb; Fig. 2a, lanes 1, 3, 4, 5) also hybridized to IS21. Since pME487 had no homology with the wild-type *P. aeruginosa* chromosome (data not shown), the 20 kb and 35 kb fragments indicate the presence of an additional IS21 copy. Previous experiments have demonstrated that in many *P. aeruginosa* Hfr strains the *trfA* gene of a chromosomally integrated, temperature-sensitive RP1 derivative is inactivated by an IS21 insertion (Reimann & Haas, 1986). This insertion may stabilize the plasmid–chromosome cointegrate by shutting off plasmid replication at any temperature. In keeping with this model, we propose that the 20 kb and 35 kb fragments originate from an IS21 insertion into the *trfA* region of the integrated pME487 (Fig. 2b). The proposed structure (Fig. 2b) is consistent with the *Cla*I digestions (Fig. 2a), but the precise location and the orientation of the internal IS21 copy have not been worked out.

Chromosomal integration of pME28

The foregoing experiments have demonstrated that a plasmid containing an IS21 tandem repeat can insert into different chromosomal sites. This result was confirmed and extended by the use of pME28, a replication-deficient RP1 derivative (Reimann & Haas, 1987). pME28 carries tandemly repeated IS21 elements adjacent to a partially deleted *trfA* gene (Fig. 1b). Hence, the plasmid depends on complementation by a functional *trfA*⁺ gene for autonomous replication (Reimann & Haas, 1987). Because of a Tra2/Tra3 deletion, pME28 is not self-transmissible but mobilization can be effected when the transfer functions are supplied *in trans*. The *E. coli* strain S17-1, which carries a chromosomally integrated IncP plasmid (Simon *et al.*, 1983), permits autonomous replication and mobilization of pME28 (Reimann & Haas, 1987). In a cross between strain S17-1(pME28) and *P. aeruginosa* PAO6049 as the recipient, *P. aeruginosa*(pME28) transconjugants were obtained at frequencies of 10^{-6} – 10^{-7} per donor; selection was made on medium containing streptomycin (3000 µg ml⁻¹), kanamycin (200 µg ml⁻¹) and tetracycline (100 µg ml⁻¹). In 10 transconjugants examined pME28 was found to be integrated into 10 different chromosomal sites, according to the variable sizes of pME28–chromosome border fragments (Fig. 3a, lanes 1, 3, 5, 8, 10, showing the patterns of five representative strains). The integrated pME28 did not contain an IS21 tandem, as judged by the absence of a 2.1 kb *Cla*I fragment (Fig. 3a, lane 12; better resolution was obtained in other experiments not shown). Hybridization with pME28 (Fig. 3a) and with an IS21 probe (not shown) indicated the presence of at least two contiguous pME28 copies in the chromosome (Fig. 3b). The formation of this structure will be considered in the Discussion.

Carbenicillin enrichment for tetracycline-sensitive PAO6049 : :pME28 derivatives resulted in the isolation of strains carrying a single chromosomal IS21 insertion. This was evident from the fact that only the IS21–chromosome border fragments hybridized to pME28 (Fig. 3a, lanes 2, 4, 6, 9, 11). Thus, the pME28 sequences had been excised, apparently by homologous recombination between the border IS21 elements (Fig. 3b).

Is pME28 insertion into the chromosome random? Whereas different insertion sites were observed in the 10 strains examined, integration was not completely random in that only two

Fig. 3. Structure of chromosomally integrated pME28 and IS21. Excision of pME28 by homologous recombination (.....) produced single IS21 insertions (see text). (a) Southern hybridization of *Cla*I-digested chromosomal DNA with 35 S-labelled pME28. Lane 1, PAO6049::pME28-9; lane 2, PAO6049::IS21-9; lane 3, PAO6049::pME28-5; lane 4, PAO6049::IS21-5; lane 5, PAO6049::pME28-17; lane 6, PAO6049::IS21-17; lane 8, PAO6049*ura*::pME28; lane 9, PAO6049*ura*::IS21; lane 10, PAO6049*trpE*::pME28 = PAO6236; lane 11, PAO6049*trpE*::IS21; lane 12, pME28 control; all digested with *Cla*I. Lane 7, pME28 digested with *Eco*RI + *Pvu*II. Band N is typically found in plasmids with IS21 tandem repeats and was lost after integration; bands F, G, H, K, L, M₁, M₂, P, Q, R are chromosome-IS21 border fragments; bands A, B, C and O are pME28-internal fragments (band O is missing from the pME28 control because of methylation of *Cla*I*); fragment C did not hybridize to an IS21 probe (not shown). (b) Proposed structure of pME28 and IS21 insertions in the *P. aeruginosa* chromosome. Letters refer to fragments in Fig. 3(a); border fragments are not shown. In one *oriT* a hypothetical insertion is indicated by dashed lines. Abbreviations are the same as in Fig. 1.

Fig. 4. Conjugational linkage in plate matings with Hfr and R68.45 donors in *P. aeruginosa*. The recipient was PAO236 in all crosses. The origins of the Hfr donors PTO6017 and PTO6034 are indicated by open arrows. Map locations are according to O'Hoy & Krishnapillai (1987). Values indicate percent linkage to unselected markers (direction of filled arrows). Linkage data from R68.45 crosses are taken from Haas & Holloway (1976).

auxotrophic mutants (one *ura* and one *trpE* mutant) could be recovered among 2700 pME28-insertional mutants of strain PAO6049.

The chromosome of strains carrying an integrated pME28 could be mobilized by an autonomous transfer-proficient helper plasmid, pME206 (=RP1 Tc^s). Hfr transfer was observed, e.g. in the *trpE*: pME28 strain PAO6236 carrying pME206. Proximal markers (*ser-3* at 19 min, *lysA* at 10 min) were transferred at 10^{-2} – 10^{-3} per donor and conjugational linkage with the multiply auxotrophic strain PAO6231 as the recipient (data not shown) confirmed that chromosome transfer originated from the *trpE* region at 22 min on the chromosome map. Transfer of the most distal chromosomal marker (*trp-6* at 23 min) occurred at 5×10^{-6} per donor. All map locations are according to O'Hoy & Krishnapillai (1987).

Conjugational linkage in matings with R68.45 or Hfr donors

In *P. aeruginosa* the piece of chromosomal DNA which is transferred during R68.45-mediated plate matings is usually short, amounting to $\leq 20\%$ of the total chromosome length (Haas & Holloway, 1978). When a derivative of RP1 or R68 was stably integrated into the chromosome as in the Hfr donors PTO6017 (Reimann & Haas, 1986) and PTO6034, longer stretches of DNA could be transferred and conjugational linkage values were higher (Fig. 4). Since the mating conditions and the recipient used were the same for all donors, the differences in linkage values must be determined by the state of the R plasmid in the donors. The simplest explanation is that the association of R68.45 with the chromosome lasts for ≤ 15 min after initiation of conjugal DNA transfer whereas stably integrated plasmids can transfer the chromosome for much longer periods.

DISCUSSION

Although R68.45 has been used extensively for chromosome mobilization in many different bacteria, the physical interaction of this plasmid with the chromosome had not been studied. The technical problem is that an R68.45-chromosome cointegrate is too unstable to be isolated, at least in *P. aeruginosa*. This instability is deduced from linkage data, such as those presented in Fig. 4. R68.45-mediated cotransfer of chromosomal markers located ≥ 15 min apart is very rare (Haas & Holloway, 1978), whereas Hfr donors can transfer the entire length of the *P. aeruginosa* chromosome (Reimmann & Haas, 1986).

The proposed tandem structure of chromosomally integrated pME28 (Fig. 3*b*) may have arisen in the following way. After insertion of one pME28 copy into the chromosome of the *rec⁺* strain PAO6049, the conditions of antibiotic selection may have favoured amplification of the resistance determinants between the directly repeated IS21 elements, by a 'transition' mechanism (Peterson & Rownd, 1985). To explain the *Cla*I fragments B (20 kb) and C (14 kb) within the integrated pME28 (Fig. 3*a*) we propose that a new *Cla*I site was created in the *oriT* region by the insertion of an unidentified element (Fig. 3*b*). The new *Cla*I site was found in all strains carrying chromosomal pME28 copies. We speculate that the presence of tandemly repeated *oriT* regions (i.e. nick sites) in the chromosome of *P. aeruginosa* may be deleterious to the host and that insertional inactivation of one *oriT* region may stabilize the integrated plasmids. We have previously observed the same kind of insertion in one *oriT* region of two chromosomally integrated pME134 [=RP1 Rep(ts)] copies in *P. aeruginosa* (Reimmann & Haas, 1986). In the case of the pME487-derived Hfr strains our model (Fig. 2*b*) shows a single plasmid copy integrated into the chromosome. From hybridizations with pME487 as the probe (not shown) a more complex structure appears also possible in that two or more interlocked pME487 copies may be present in the chromosome, again with insertional inactivation of *trfA* and one *oriT*. The main conclusion from all hybridization experiments, however, is that chromosomal copies of pME487 or pME28 do not contain any IS21 duplication.

The fact that chromosomally integrated pME487 and pME28 were bordered by single IS21 elements is in agreement with a model which pictures R68.45-like plasmids as large, circular transposons using a 'cut-and-paste' mode of transposition (Reimmann & Haas, 1987).

In previous work it was suggested that R68.45 could cause insertional mutations in the *P. aeruginosa* chromosome but no physical evidence was provided (Holloway *et al.*, 1979). Our present study indicates that the R68.45-like plasmid pME28 does have mutagenic properties. Although IS21 insertions occurred in many different chromosomal sites, the target sequence cannot be totally random, as shown by the low frequency of pME28-induced auxotrophic mutations in *P. aeruginosa*.

The use of pME28 as an *oriT⁺* transposon similar to Tn5-Mob (Simon, 1984) is illustrated by the example of the Hfr donor PAO6236(pME206). It is also feasible to insert pME28 into another plasmid, which thus becomes mobilizable by RP1 (M. Gamper, personal communication). Occasionally, we have encountered mobilization-deficient pME28 insertions in the *P. aeruginosa* chromosome, perhaps as a consequence of secondary rearrangements within pME28 sequences but this problem did not arise in pME28-plasmid cointegrates (our unpublished results). Strains PAO6236(pME206) and PTO6034 can be added to the collection of Hfr donors now available for rapid chromosome mapping in *P. aeruginosa* PAO (Haas *et al.*, 1981; O'Hoy & Krishnapillai, 1985; Reimmann & Haas, 1986; O'Hoy & Krishnapillai, 1987).

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