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Transfer of Plasmid RP4 to *Myxococcus xanthus* and Evidence for Its Integration into the Chromosome

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The broad-host-range plasmid RP4 and its derivative R68.45 were transferred to *Myxococcus xanthus* DK101 and DZ1; RP4 was maintained integrated in the chromosome. Loss of plasmid markers occurred during the growth of the transconjugants, which could be prevented by selective pressure with oxytetracycline. The integrated plasmid was transferred back to *Escherichia coli* often as RP4-prime plasmids carrying various segments of the *M. xanthus* chromosome. It also mediated chromosomal transfer between *M. xanthus* strains.

The bacterium *Myxococcus xanthus* has several remarkable traits. It is able to aggregate and differentiate into multicellular fruiting bodies (21) and produces antibiotics (29, 39) and proteins (26a) which are excreted into the surrounding medium. Currently, genetic manipulations use coliphage P1 to transfer *Myxococcus xanthus* genes previously cloned via pBR322 in *Escherichia coli* back to *M. xanthus* (28, 34). Although numerous phages of *M. xanthus* are known (21), no endogenous plasmid has been found, and few attempts to transfer exogenous plasmids have been reported (24, 30).

The plasmids of the P1-Inc group have a broad host range (7), being transferable to a wide variety of gram-negative bacteria. Some of them have been reported to mediate chromosome transfer (18, 37) or R-prime plasmid formation (17, 19, 23, 26). The most frequently used plasmid RP4 (32) promotes chromosomal transfer in a limited number of cases (2, 36). Therefore, different strategies have been designed to enhance chromosomal integration of RP4: (i) in vitro construction of an RP4-prime plasmid to allow integration by genetic recombination (3, 20); (ii) selection of a temperature-sensitive RP4 for maintenance (14), and (iii) use of transposable elements that mediate RP4 integration in the chromosome by cointegrate formation (19, 38). R68.45, which is an RP4 plasmid exhibiting a duplicated IS*21* (12, 13), originally unique in RP4, falls into this class.

In this paper, we show that RP4 can be transferred into *M. xanthus* where it is maintained integrated into the chromosome. It promoted the transfer of chromosomal markers between *M. xanthus* strains and R-prime plasmids when transferred back to *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *M. xanthus* was grown in CYE medium (Casitone [Difco Laboratories], 1%; yeast extract, [Biomerieux], 0.5%; $MgSO_4$, 0.1%; pH 7.6) at 30°C. *E. coli* was grown in L broth (25) at 37°C. These media were solidified with 1.2% agar (Biomerieux).

For *M. xanthus*, the antibiotics used and final concentrations (in milligrams per milliliter) were: kanamycin sulfate, 75 (Bristol Laboratories); oxytetracycline chloride, 12.5 (Roussel-Uclaf); carbenicillin, 500 (Beecham-Sevigne); nalidixic acid, 100 (Sigma Chemical Co.), rifampin, 25 (Lepetit); streptomycin sulfate concentration as given below (Diamant). For *E. coli*, the antibiotics and their concentrations (in milligrams per milliliter) were: kanamycin sulfate, 25; tetracycline chloride, 10 (Roussel-Uclaf); ampicillin, 25 (Bristol); nalidixic acid, 100.

Matings. (i) *E. coli* as donor. A stationary-phase culture of *M. xanthus* (500 μl; 10^9 cells) and 2 ml of an unshaken culture suspension of log-phase *E. coli* carrying the plasmid were spun down together, suspended in 200 μl of L broth, and incubated at 30°C overnight on a membrane filter placed onto an L plate. The cells were resuspended in 5 ml of CYE medium and shaken at 30°C for 4 h, and appropriate dilutions were spread in soft agar onto selective media (kanamycin, 75 mg/ml). The donor strains were counterselected with streptomycin.

(ii) *M. xanthus* as donor. *M. xanthus* cells carrying the plasmid markers were picked onto solid medium containing the appropriate antibiotics and grown at 30°C for 36 to 48 h. Cells from about two colonies were mixed with 200 μl of a stationary-phase culture of either *E. coli* or *M. xanthus* and filter mated as described above on CYE solid medium. The cells were suspended in either 5 ml of L broth (*E. coli* recipient) or 5 ml of CYE medium (*M. xanthus* recipient). The donor strain was counterselected by nalidixic acid or rifampin or both.

Test for stability and phenotype of transconjugants. Km^r transconjugants were purified by streaking a colony onto a medium without antibiotics; individual colonies were then picked onto appropriate selective plates. The *tet* locus of RP4 specifies resistance to tetracycline in *E. coli* and to oxytetracycline in *M. xanthus*. The resistance conferred by the *bla* locus (transposon Tn*1*) is difficult to handle. All strains of *M. xanthus* were resistant to at least 500 mg of ampicillin per liter and to at least 200 mg of pennicillin G per liter. Strain DK101 and derivatives were resistant to 500 mg of carbenicillin per liter, and the presence of RP4 did not substantially increase these levels of resistance. However, DZ1 was sensitive to carbenicillin, and the presence of the RP4 Ap/Cb marker could be detected by 500 mg of that antibiotic per liter. The instability of this antibiotic and the slow growth of *M. xanthus* (2 to 4 days at 30°C for colonies transferred by picking) necessitate care in the preparation of the stock and the use of positive and negative controls of growth.

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TABLE 1. Strains and plasmids

Strain	Relevant characteristics	Source (reference)
M. xanthus		
DK101	Reference wild type	Hodgkin and Kaiser (16)
DZ1[a]	Str^r	Zusman et al. (40)
CM207	Str^r spontaneous isolate from DK101	This work
CM213	Nal^r Rif^r, two-step spontaneous isolate from DK101	This work
CM1011	One clone, DK101(RP4) Km^r Tc^r Cb^r	This work
CM2003	One clone, DZ1(RP4) Km^r Tc^r Cb^r	This work
Pooled ΩTn*5*	Pool of random chromosomal Tn*5* insertions (Ω) isolated from DK101 Km^r Str^r	Nicaud et al. (26a)
E. coli		
W3101	*recA13 trpE* Nal^r	Kopecko et al. (22)
W3101(RP4)	id + Km^r Tc^r Ap^r	Hassan and Brevet (15)
W3101(R68.45)	Obtained by transfer of R68.45 from PAO25	This work
W3101(RP4 *kan-2*::Tn*7*)	*recA13 trpE* Rif^r Km^s Tc Ap	Hassan and Brevet (15)
P. aeruginosa PAO25(R68.45)	Str^r Km^r Tc^r Ap^r	Haas and Holloway (12)
RP4-prime plasmids		
pCM2003-6	Km^r Tc^r Ap^r, different-molecular-weight plasmids obtained by transfer of RP4 from CM2003 to W3101 *recA13 trpE* Nal^r	This work
pCM2003-7		This work

[a] DZ1 is a strain of *M. xanthus* widely used because it produces less mucus and is a good acceptor for myxophages (it behaves like an *hsdS* mutant). Km, Tc, and Ap/Cb are plasmid markers described by Novick et al. (27).

Plasmid detection. Free supercoiled plasmid DNA was visualized by the in-gel lysis method of Eckhardt (11). The cells were taken directly either from colonies on plates or from liquid culture. About 10^8 to 2×10^8 cells were used for each well.

Plasmid extraction. RP4 to be used as a probe was extracted from *E. coli* W3101(RP4) by the clear-lysate technique (6), and then the supercoiled plasmid DNA was separated from linear chromosomal DNA by ultracentrifugation in a cesium chloride-ethidium bromide gradient (5). After removal of the ethidium bromide, the DNA was dialyzed against TE buffer (Tris, 10 mM; trisodium EDTA, 1 mM; pH 8).

Preparation of DNA and Southern blot hybridization. The blot hybridization method developed by Southern (35) was used to determine the numbers and the sizes of *M. xanthus* chromosomal DNA fragments homologous to RP4. The chromosomal DNA was extracted as described by Davis et al. (8) adapted for *M. xanthus* by Avery and Kaiser (1); 5 μg of DNA was digested by the restriction enzyme *Sal*I as described by the supplier (Boehringer-Mannheim). Lambda DNA digested by *Eco*RI (Boehringer-Mannheim) was used as a standard, Tris-acetate agarose gels were run, and DNA was transferred from the gels to nitrocellulose as described before (8). The RP4 DNA to be used as a probe was labeled by nick translation, using 2 mCi of [α-^{32}P]dCTP (Amersham Corp.) per ml and 200 ng of DNA in 25-μl volumes incubated at 15°C by the Rigby et al. method (31).

To hybridize the labeled probe to the chromosomal DNA fragments attached to nitrocellulose, we used a slightly modified Denhardt method (9). The hybridization buffer contained 10 times the preincubation buffer used by Denhardt (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate). The prehybridization lasted for 4 h at 68°C and the hybridization in this same buffer with the denatured labeled RP4 lasted for 16 h at 68°C, which was followed by four washes of the filter with 3× SSC–0.2% sodium dodecyl sulfate at 68°C and two washes with 0.5× SSC at 68°C. The bands hybridized to the labeled probe were revealed by autoradiography as described before (8).

RESULTS

Transfer of RP4 and R68.45 from *E. coli* to *M. xanthus*. The transfer of plasmids RP4 and R68.45 from *E. coli* to *M. xanthus* occurred at rather low frequencies (Table 2). There was a drastic difference in transfer frequencies between recipient strains of *M. xanthus*. The transfer of RP4 occurred 10^5 times more frequently in strain DZ1 than in strain DK101, and the transfer of R68.45 was increased by a factor of 10^2 in DK101 as compared with transfer of RP4. The phenotype of the transconjugants varied widely with regard to the size and color of the colonies.

Stability of transconjugants. Transconjugants resistant to kanamycin from the cross DZ1 × W3101(RP4) were studied for their antibiotic resistances either directly from the selection plates or after purification on antibiotic free medium (Table 3). Only one-third of the transconjugants expressed the three antibiotic resistances conferred by RP4. More than 50% lost Cb^r, and 9% lost both Cb^r and Tc^r; 2% lost only Tc^r (Table 3). When transconjugants from each class were purified on antibiotic-free medium, the data confirmed the instability of the markers and the fact that Cb^r was lost preferentially, and then Tc^r and eventually Km^r were lost (Table 3). The Cb marker was found in purified colonies obtained from clones of transconjugants that appeared previously to be Cb^s (Table 3). This, and the presence of sectors on some replica plates indicated that segregation was not established at once, but continued along with cell division, and colonies were probably a mixed population of cells.

In agreement with these findings, we were able to maintain stable transconjugants by selective pressure with oxytetracycline (we could not use carbenicillin because of the difficulties outlined above). So far, and after a great number

TABLE 2. Plasmid-mediated transfers

Recipient × donor	Transconjugant frequency[a]	Resistance selection	Spontaneous resistance frequency	
			Recipient	Donor
E. coli as donor				
DK101[b] × W3101(RP4)	1×10^{-8}–5×10^{-8}	Km Str[c]	$<10^{-10}$	NR[d]
DZ1 × W3101(RP4)	3×10^{-3}–6×10^{-3}	Km Str[e]	$<10^{-10}$	NR
CM207 × W3101(R68.45)	9×10^{-6}	Km Str[e]	$<10^{-10}$	NR
DZ1 × W3101(R68.45)	8×10^{-4}	Km Str[e]	$<10^{-10}$	NR
M. xanthus as donor				
W3101 Nal × CM1011	4.4×10^{-6}	Nal Km Tc Ap	$<1.6 \times 10^{-9}$	NR
W3101 Nal × CM2003	8.3×10^{-4}	Nal Km Tc Ap	$<1.6 \times 10^{-9}$	NR
CM213 × pooled ΩTn*5*(RP4 *kan-2*::Tn*7*)	1×10^{-8}	Km Nal Rif	$<10^{-10}$	$<10^{-12}$

[a] Compared with the donor. In heterologous matings, the frequency was calculated as compared with viable myxococci, with *E. coli* being always in excess.
[b] Either DK101 or the Str[r] CM207 derivative was used in this cross.
[c] Streptomycin, 20 μg/ml.
[d] NR, Not relevant. Colonies of *E. coli* and *M. xanthus* were easily distinguishable on the plates; therefore, the scarce, spontaneously resistant colonies that may contaminate the plates were ignored.
[e] Streptomycin, 100 μg/ml.

of generations on this medium, we have not found evidence of a single Km[s] Tc[r] segregant among the *M. xanthus* transconjugants bearing RP4.

Evidence for integration of plasmid RP4 into the *M. xanthus* chromosome. Three points were investigated. (i) We could not demonstrate the presence of the free plasmid in the transconjugants by agarose gel electrophoresis (technique of Eckhardt [11]). No band corresponding to the free supercoiled plasmid could be seen in lysates from *M. xanthus* transconjugants bearing the three RP4-carried resistances, in contrast to the control RP4 donor W3101(RP4). Moreover, the same results were obtained when the experiments were performed with nascent transconjugants or with transconjugants harboring the three resistance markers and grown under selective pressure. To rule out any experimental artifact that might be due to active DNases, for example, the cells were heated (10 min at 60°C) before lysis. This treatment did not allow the recovery of any plasmid band in *M. xanthus* transconjugants. In addition, mixing cells of W3101(RP4) and *M. xanthus* in the same well before lysis and electrophoresis did not prevent the appearance of supercoiled RP4 band.

(ii) We did a Southern blot hybridization of the chromosome of transconjugant CM1011 with labeled RP4. Figure 1 shows that material hybridizing with RP4 in the undigested chromosomal DNA had a higher molecular weight than did the free RP4. The *Sal*I-digested RP4 displayed two bands of 24.5 and 11.5 megadaltons as previously shown (10), and the digested DNA of strain CM1011 displayed three bands of 24, 13, and 6 megadaltons, respectively, the total giving a molecular mass greater than that of RP4.

(iii) We performed matings with *M. xanthus* transconjugants as donors. Table 2 shows the markers transferred and the frequencies observed. When *E. coli* was the recipient, the markers carried by the RP4 plasmid were cotransferred back to *E. coli* either from strain CM1011 or CM2003.

We demonstrated the plasmids obtained in these second generation transconjugants by agarose gel electrophoresis; with strain CM1011 as a donor, the plasmids of all the transconjugants tested (about 10) migrated at the same level as did the original RP4 (data not shown), whereas when CM2003 was the donor, 9 out of 10 migrated more slowly and therefore had a higher molecular weight than did RP4. Some representatives are shown in Fig. 2.

The sizes of these plasmids were not only larger than that of RP4 but were different compared with one another. One of these plasmids (pCM2003-6; Fig. 2, lane 2) was extracted and restricted as shown in Fig. 3. In addition to the bands relevant to RP4 digestion, several additional bands appeared. Another plasmid (pCM2003-7; fig. 2, lane 3) of higher molecular weight displayed the same pattern as that of pCM2003-6 with some more additional bands. Therefore, these derivatives were R-prime plasmids carrying fragments of chromosomal *M. xanthus* DNA of different sizes.

We also performed homologous matings. In a cross, the donor was a pool of random chromosomal Tn*5* insertions to which the plasmid RP4 *kan-2*::Tn*7* (Km[s]) had been transferred, and the receiver was an Nal[r] Rif[r] derivative of

TABLE 3. Instability of transconjugants from a D21 × W3101 Nal(RP4) cross

Phenotype of transconjugants and their segregants	Distribution of 100 Km[r] transconjugants	No. (%) of segregants[a]:			
		Km[r] Tc[r] Cb[r] (n = 7)[b]	Km[r] Tc[r] (n = 6)	Km[r] (n = 4)	Km[r] Cb[r] (n = 2)
Km[r] Tc[r] Cb[r]	36	36 (46)	6 (17)	0	0
Km[r] Tc[r]	53	35 (44)	21 (60)	0	0
Km[r]	9	7 (9)	8 (23)	3	0
Km[r] Cb[r]	2	1 (1)	0	9	0
Cb[r]	0	0	0	2	7
No resistances	0	0	0	14	5

[a] Segregants were obtained from purified transconjugants as described in the text. The sum and percentage of segregants arising from one class exhibiting one considered phenotype are shown.
[b] Number of transconjugants purified in each class; variable numbers of purified colonies were tested from each.

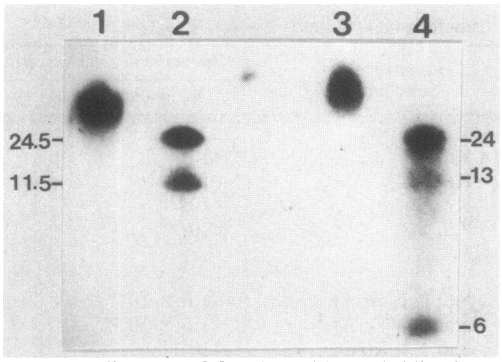

FIG. 1. Autoradiogram of Southern blot hybridization with an RP4 probe. Lanes: 1, W3101(RP4) DNA; 2, W3101(RP4) DNA digested by *Sal*I; 3, CM1011 DNA; 4, CM1011 DNA digested by *Sal*I. Sizes of DNA fragments are shown in megadaltons.

DK101(CM213) (Table 2). The transfer of Tn*5* from the donor to the recipient without cotransfer of the Tc marker of RP4 could be shown unambiguously since Tn*5* confers on *M. xanthus* both Km^r and Str^r (4). The frequency of transfer was very low yet indicative of chromosomal transfer between *M. xanthus* strains.

DISCUSSION

Our data demonstrated that the broad-host-range plasmid RP4 can be transferred from *E. coli* to *M. xanthus* with frequencies varying from 10^{-8} to 10^{-3} depending on the recipient strain. RP4 could be maintained in *M. xanthus* but was not visualized as a free plasmid. Integration of RP4 into the host chromosome was indicated by the results of the hybridization experiment. An RP4 probe that hybridized with two *Sal*I fragments of the free RP4 hybridized with three *Sal*I fragments of the chromosome of strain CM1011 [DK101(RP4)].

The difference between strain DZ1 and strain DK101 with respect to RP4 transfer was striking. Neither strain was able to self-replicate RP4, but the frequencies of transfer were 10^5 times higher in DZ1. Also, plasmid R68.45 bearing two IS*21* sequences, which is known to integrate in the chromosome of several gram-negative species at rather high frequencies (18, 23, 38), did not transfer to strain DZ1 more efficiently than did RP4, whereas R68.45 transferred 10^2 times more efficiently in strain DK101 than did RP4. These differences may come from physiological differences; strain DZ1 produces less slime, less lytic enzymes (that may kill the donor strain), and fewer or no restriction enzymes. They may also come from differences in the integration mechanism of RP4 into the chromosome of different strains. Experiments in progress will hopefully shed light on this point.

Another argument in favor of the integration of RP4 into the *M. xanthus* chromosome which may prove to be of great genetic interest is that it can be transferred back to *E. coli*, where it replicates as a free plasmid often larger than the original RP4. We showed that they were RP4-prime plasmids carrying various lengths of the *M. xanthus* chromosome. In addition, the fact that the phenotypes of the transconjugants varied widely tended to show that RP4 integrated in different points of the *M. xanthus* chromosome. This is the first evidence of the possibility of in vivo cloning of *M. xanthus* DNA into *E. coli*. This also may provide a simple way to perform complementation tests in *M. xanthus*.

Also, the transfer of a transposon from one *M. xanthus* strain to another without transfer of the accompanying RP4 markers suggested that chromosomal transfer had occurred. This property considerably enlarges the scope of genetic manipulation of *M. xanthus*, although frequencies were still low with DK101 and derivative strains, in spite of an instability of the RP4 markers when the cells were grown on antibiotic-free medium.

The marker loss appeared to be well ordered. Ap^r was lost most frequently, followed by Tc^r; Km was lost only when the two other markers were lost, with the exception of few

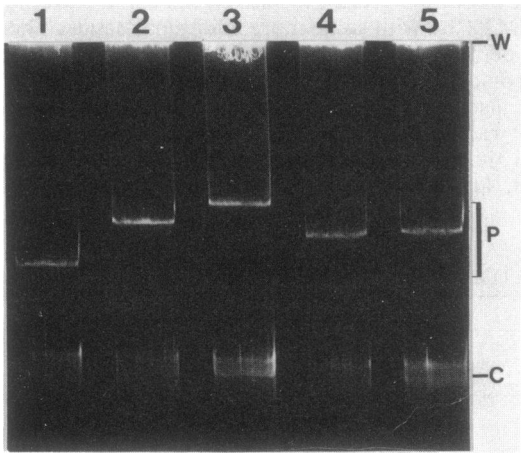

FIG. 2. Electropherograms of RP4 and RP4 derivative plasmids obtained in *E. coli* transconjugants wherein the donor was the clone CM2003. Lanes: 1, W3101(RP4); 2, pCM2003-6; 3, pCM2003-7; 4 and 5, other clones of *E. coli* transconjugants obtained by transfer of RP4 from strain CM2003. W, well; P, plasmid band; C, chromosomal band.

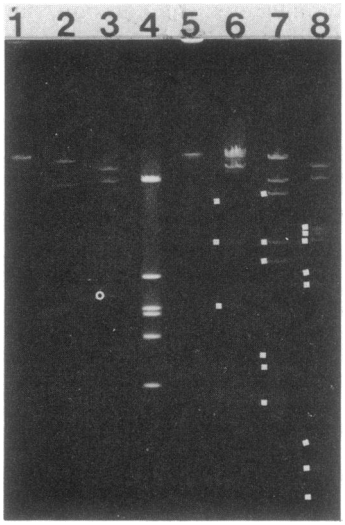

FIG. 3. Restriction analysis of RP4 and the RP4 derivative plasmid pCM2003-6. Lanes: 1 to 3, RP4 digested by *Eco*RI, *Sal*I, and *Pst*I, respectively; 4, λ markers; 5 to 8, undigested pCM2003-6 and pCM2003-6 digested by *Eco*RI, *Sal*I, and *Pst*I, respectively. Symbols: ○, band that disappeared from the RP4 pattern; □, bands that appeared in the pCM2003-6 pattern. Plasmids were extracted by the clear-lysate technique as described in the text.

segregants wherein Cb^r was retained, which can be explained by transposition of Tn*1*. The transconjugants could be stabilized by growing them on oxytetracycline-containing medium. This marker loss did not take place immediately, in contrast with that observed when RP4 and RK2 are transferred into *Alcaligenes eutrophus* (33). The process we studied was similar to that described by Haas and Riess for *Pseudomonas aeruginosa* (13). Upon transfer of R68.45, they showed that sequential deletion of the plasmid started from IS*21*, through which integration had occurred. With DZ1 transconjugants, the order of the loss of the markers suggested that deletion starts from a point of the plasmid other than on IS*21*. However, at least one DK101 transconjugant was found which appeared completely stable. This might be extremely valuable for further genetic studies with RP4. A study of transconjugant stability with respect to the recipient strain and the site(s) of insertion into the chromosome is in progress in our laboratory.

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