JOURNAL OF BACTERIOLOGY, Mar. 1990, p. 1663–1666
0021-9193/90/031663-04$02.00/0

Vol. 172, No. 3

High-Frequency Conjugal Plasmid Transfer from Gram-Negative *Escherichia coli* to Various Gram-Positive Coryneform Bacteria

ANDREAS SCHÄFER, JÖRN KALINOWSKI, REINHARD SIMON, ANNA-HILDEGARD SEEP-FELDHAUS, AND ALFRED PÜHLER*

Department of Genetics, University of Bielefeld, Postbox 8640, 4800 Bielefeld, Federal Republic of Germany

Received 31 July 1989/Accepted 7 December 1989

We report on the mobilization of shuttle plasmids from gram-negative *Escherichia coli* to gram-positive corynebacteria mediated by P-type transfer functions. Introduction of plasmids into corynebacteria was markedly enhanced after heat treatment of the recipient cells. High-frequency plasmid transfer was also observed when the restriction system of the recipient was mutated. On the basis of our data, we conclude that efficient DNA transfer from gram-negative to gram-positive bacteria, at least to coryneform bacteria, is conceivable in certain natural ecosystems.

Conjugal transfer of broad-host-range IncP-type resistance plasmids within gram-negative bacterial species is well known (3, 9, 18). Non-self-transmissible plasmids carrying the appropriate origin of transfer (*oriT*) can be mobilized by IncP plasmids (28). Recent studies have shown that conjugation is a nonspecific process and accounts for most horizontal gene transfer between even phylogenetically remote organisms (7, 13, 25, 26).

To investigate the possibility of conjugal plasmid transfer between *Escherichia coli* and *Corynebacterium glutamicum*, we took advantage of a mobilization system previously developed for genetic engineering of a wide range of gram-negative bacteria (23). This strategy is based on the *oriT* and the transfer (Tra) functions of IncP-type broad-host-range plasmid RP4 (10) and consists of *E. coli* mobilizing strains and derivatives of conventional *E. coli* vectors (pSUP vectors [22, 23]). A series of *E. coli-C. glutamicum* shuttle plasmids was constructed based on mobilizable *E. coli* vector pSUP102 (22). The 10.6-kilobase prototype shuttle vector pECM1 resulted from fusion of pSUP102 to *C. glutamicum* vector pCV35 (Fig. 1).

Transfer of pECM1 to *C. glutamicum*. For mating experiments, plasmid pECM1 was introduced by transformation into mobilizing strain *E. coli* S17-1 (23). *E. coli* S17-1 carries an RP4 derivative integrated into the chromosome which provides the transfer functions necessary for mobilization. By using this donor, plasmid pECM1 was transferred by conjugation as previously described (23) to a nalidixic acid-resistant derivative of *E. coli* MM294 (14) at frequencies between 10^{-1} and 10^{-2} (Table 1) per donor cell. For conjugal transfer of pECM1 to coryneform recipient strains, donor strain S17-1(pECM1) was grown to the late-exponential phase in LB medium (15) containing 50 μg of kanamycin per ml. Recipient strains were grown in LB medium to an optical density at 580 nm of 3 to 4. About 7×10^8 donor and 3.5×10^9 recipient cells, corresponding to a ratio of 1:5, were mixed and pelleted by centrifugation at 20°C for a short time. The mating mixture was then carefully suspended in about 500 μl of LB medium and spread onto a 0.45-μm-pore-size cellulose acetate filter (Millipore Corp., Bedford, Mass.) placed on a prewarmed LB plate. After 20 h of incubation at 30°C, the cells were washed from the filter with 1 ml of LB medium and mechanical agitation. Transconjugants were selected by plating serial dilutions on LB medium containing 25 μg of kanamycin per ml and 30 μg of nalidixic acid per ml. All of the coryneform strains listed in Table 2 showed natural resistance to at least 30 μg of nalidixic acid per ml. To confirm the presence of pECM1, 24 transconjugants of each strain were lysed as described elsewhere (5) and their plasmid contents were analyzed by agarose gel electrophoresis. Mobility and restriction patterns of the isolated plasmid DNAs from transconjugants were indistinguishable from those obtained with pECM1 from *E. coli* S17-1 (data not shown). Transfer frequencies are expressed as the number of transconjugants per final donor colony.

Under optimal mating conditions, *C. glutamicum* recipient cells acquired pECM1 at a frequency of 10^{-6} to 10^{-7} (Table 1). Alternative transfer mechanisms were excluded by performing control matings (Table 1). No transmission of pECM1 occurred when *E. coli* C600 (1), a nonmobilizing strain, was used as the donor. No *C. glutamicum* transconjugants were detected when plasmid pEC1, consisting of plasmids pACYC184 (8) and pCV35 fused at their *Bam*HI sites and lacking mobilization functions, was used in the mobilization experiment. There was no transfer of pECM1 in control experiments using cell-free supernatant of the donor culture or purified plasmid DNA instead of donor cells. Moreover, the presence of 70 μg of DNase I (Sigma Chemical Co.) per ml in the mating mixture did not affect transfer efficiency. The results fit the current definition of conjugation, ruling out transformation and transduction as possible transfer mechanisms.

Heat treatment of recipient cells. An effective restriction system might be responsible for low fertility in intergenic matings (11, 17). We were able to block such a possible restriction system by heat treating the recipients before mating them, resulting in a dramatic increase of fertility (Table 1). When heat treatment was applied for 9 min, the minimum temperature required to raise the transfer frequency in subsequent intergenic matings was 42.5°C (Fig. 2a). A maximum of 10^7 to 10^8 transconjugant colonies, corresponding to transfer frequencies between 10^{-2} and 10^{-3}, were obtained after incubation of *C. glutamicum* at 48.5°C (Table 1; Fig. 2a). Heat treatment at temperatures higher than 50°C led to lower transfer frequencies, probably due to decreasing viability of the recipient cells.

The persistence of high competence after heat incubation of *C. glutamicum* was tested (Fig. 2b). When recipient cells

* Corresponding author.

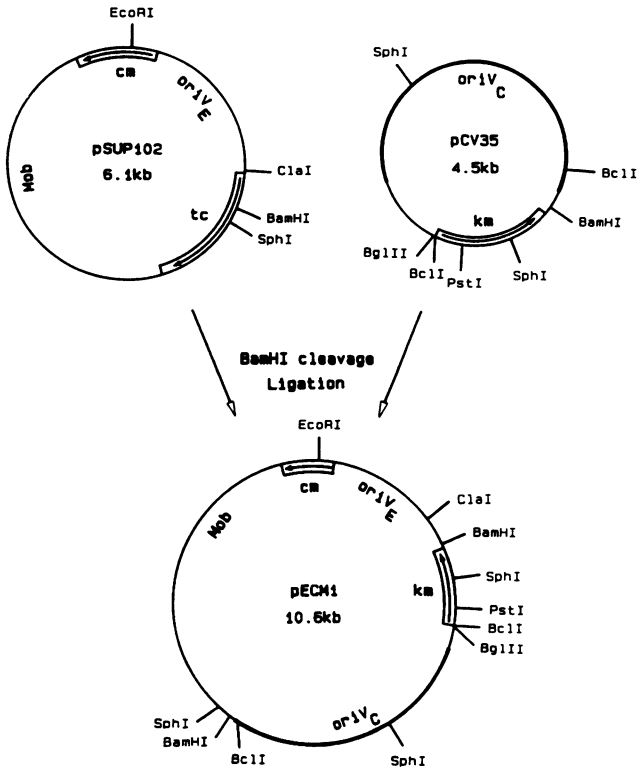

FIG. 1. Construction of mobilizable shuttle vector pECM1. Vector pCV35 is based on *C. glutamicum* replicon pHM1519 (16) and was constructed in our laboratory (unpublished data). pECM1 carries the RP4 mobilization fragment (Mob), which contains the origin of transfer replication, the origins of vegetative replication in *E. coli* ($oriV_E$) and *C. glutamicum* ($oriV_C$), and chloramphenicol (cm) and kanamycin (km) resistance determinants. The latter determinant is derived from transposon Tn*5* and is known to be expressed in *E. coli* and coryneform bacteria (19). The bold line marks the pHM1519 replicon. kb, Kilobases.

were grown in fresh medium at 30°C after heat treatment, the enhancing effect declined within 5 h after heat incubation. Heat-treated recipient cultures stored at 30°C without addition of fresh medium to prevent further growth retained their competence for at least 48 h.

Heat treatment affects the restriction system. To examine

TABLE 2. Mobilization of *E. coli-C. glutamicum* shuttle vector pECM1 from *E. coli* S17-1 to different strains of coryneform bacteria[a]

Coryneform recipient strain[b]	Transfer frequency
Group A	10^{-2}–10^{-4}
Arthrobacter albidus DSM 20128	
Brevibacterium divaricatum DSM 20297	
Brevibacterium lactofermentum DSM 20412	
Brevibacterium roseum ATCC 13825	
Corynebacterium callunae DSM 20147	
Corynebacterium glutamicum ATCC 13032	
Corynebacterium glutamicum ATCC 13058	
Corynebacterium herculis DSM 20301	
Group B	10^{-5}–10^{-7}
Brevibacterium ammoniagenes DSM 20305	
Brevibacterium flavum DSM 20411	
Brevibacterium stationis DSM 20302	
Corynebacterium acetoacidophilum ATCC 13870	
Corynebacterium ilicis DSM 20138	
Corynebacterium lilium DSM 20137	
Corynebacterium pilosum DSM 20521	
Group C	$<10^{-8}$
Corynebacterium fascians DSM 20131	
Corynebacterium melassecola ATCC 17965	
Corynebacterium melassecola ATCC 17966	
Group D	No transconjugants detected
Brevibacterium imperiale DSM 20530	
Brevibacterium linens DSM 20158	
Brevibacterium luteum DSM 20542	
Brevibacterium ketoglutamicum DSM 20165	
Brevibacterium pusillum DSM 20527	
Brevibacterium testaceum DSM 20166	
Corynebacterium flaccumfaciens DSM 20129	
Clavibacter michiganense NCPPB 382	
Clavibacter nebraskense NCPPB 2581	
Corynebacterium xerosis DSM 20170	

[a] The mating procedure used is described in the text. At least three independent matings per strain were performed. The results were reproducible within 1 order of magnitude.

[b] DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection; NCPPB, National Collection of Plant Pathogenic Bacteria.

TABLE 1. Conjugal transferability of shuttle vectors from *E. coli* to *C. glutamicum*[a]

E. coli donor	Recipient	Heat treatment of recipient for 9 min (48.5°C)	DNase I treatment of mating mixture (70 μg/ml)	Transfer frequency/donor
S17-1(pECM1)	*E. coli* MM294 (Nx^r)[b]	No	No	6×10^{-2}
S17-1(pECM1)	*C. glutamicum* ATCC[c] 13032	No	No	4×10^{-7}
S17-1(pECM1)	*C. glutamicum* ATCC 13032	Yes	No	5×10^{-3}
S17-1(pECM1)	*C. glutamicum* RM3 (Res^-)	No	No	1×10^{-2}
S17-1(pECM1)	*C. glutamicum* RM3 (Res^-)	Yes	No	7×10^{-3}
S17-1(pECM1)	*C. glutamicum* ATCC 13032	Yes	Yes	5×10^{-3}
C600(pECM1)	*C. glutamicum* ATCC 13032	Yes	No	$<10^{-9}$
S17-1(pEC1)	*C. glutamicum* ATCC 13032	Yes	No	$<10^{-9}$
Donor supernatant	*C. glutamicum* ATCC 13032	Yes	No	$<10^{-9}$
Purified pECM1 DNA	*C. glutamicum* ATCC 13032	Yes	No	$<10^{-9}$

[a] The mating procedure was performed as described in the text. The values shown are means of three independent matings and were reproducible within 1 order of magnitude.

[b] Nx^r, Resistance to nalidixic acid at 50 μg/ml.

[c] ATCC, American Type Culture Collection.

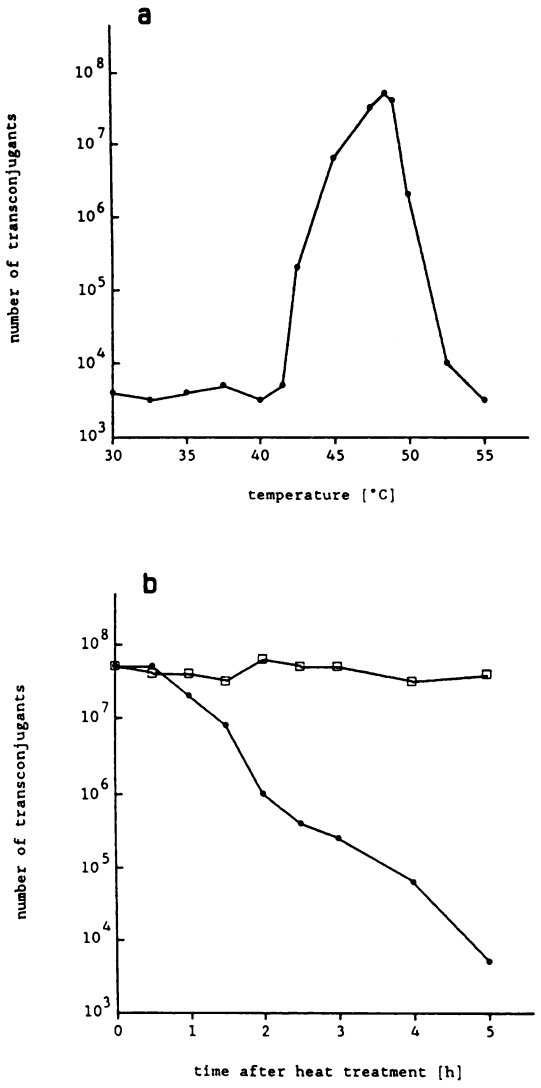

FIG. 2. Temperature dependence of transfer frequency and persistence of heat treatment response. (a) Heat treatment of recipient cells was performed by the mating protocol for 9 min at various temperatures. (b) Persistence of increased fertility after heat treatment in growing (●) and nongrowing (□) recipient cultures. Three independent matings were performed. Values were reproducible within 1 order of magnitude.

the influence of the restriction system of the recipient cells on conjugal transfer of pECM1, we took advantage of a restriction-deficient (Res^-) mutant of *C. glutamicum* ATCC 13032 isolated in our laboratory after nitrosoguanidine mutagenesis. This mutant, designated *C. glutamicum* RM3, was transformable with pECM1 DNA isolated from *E. coli*. By using polyethylene glycol-mediated spheroplast transformation (24), up to 10^3 transformants per μg of plasmid DNA were obtained. No transformants were detected after transformation of *C. glutamicum* ATCC 13032. Interestingly, we found high-frequency conjugal transfer of pECM1 into Res^- *C. glutamicum* cells with no preceding heat treatment (Table 1). In addition, no further increase in transfer efficiency was noticed when *E. coli* S17-1(pECM1) was mated with preheated Res^- *C. glutamicum* cells. We therefore suggest that heat treatment impairs the restriction of incoming foreign DNA by recipients.

Transfer to various strains of coryneform bacteria. Conjugal transfer of pECM1 was not restricted to the recipient *C. glutamicum* (Table 2). By the described mating protocol, we mobilized pECM1 from *E. coli* S17-1 to 28 preheated wild-type strains of coryneform bacteria. With regard to the transfer frequency, the strains tested were divided into the four groups (A to D) listed in Table 2. All of the group A and most of the group B and C strains are classified as members of the so-called *C. glutamicum-Brevibacterium ammoniagenes* cluster (21). Most of these strains are physiologically extremely homogeneous and are supposed to be identical or closely related to *C. glutamicum* (6, 21). Group D strains are taxonomically more heterogeneous, and none of them is thought to be closely related to *C. glutamicum* (6, 21). Plasmid pECM1 could not be transferred by conjugation from *E. coli* to any of the group D strains. However, there is evidence that conjugal transfer of shuttle vectors into group D strains is achievable when suitable replicons are applied. Shuttle vectors based on a *B. linens* replicon were successfully mobilized from *E. coli* S17-1 to *B. linens* by using the protocol described here (unpublished data). Although *B. linens* is of interest to the cheese-producing industry, there has previously been no method available for introduction of plasmid DNA into this strain.

Conclusions. We have demonstrated that conjugal transfer of plasmid DNA from gram-negative *E. coli* to gram-positive coryneform bacteria is possible. Although procedures for transformation of spheroplasts and protoplasts have been developed recently (24), there has been no method available for efficient introduction of foreign plasmid DNA into corynebacteria. This is the first report of high transfer frequencies resulting either from heat treatment of recipient cells before mating or, alternatively, from using a restriction-deficient mutant of *C. glutamicum*. We suggest that heating blocks the restriction system of recipient cells. High temperatures might act in two ways to impair the restriction function: either directly by inactivating temperature-sensitive restriction endonucleases (2, 12, 17) or indirectly, like UV radiation (4, 20, 27), in the course of SOS repair as a consequence of DNA damage. The results reported suggest that the restriction system of the recipient cells is the main barrier preventing efficient conjugal transfer between *E. coli* and a wide range of coryneform strains. It is conceivable that this result is applicable to other gram-positive genera, like *Staphylococcus*, *Streptococcus*, *Bacillus* (26), or *Streptomyces* (13). However, it remains unclear why impairment of the restriction system raises the mating efficiency, since it is generally believed that single-stranded transfer, as it probably occurs in the system described here, is insensitive to restriction in the recipient.

Use of the conjugal transfer system reported here should greatly facilitate optimization of amino acid production by *C. glutamicum* and related species, since *E. coli* recombinant DNA technology can now be applied to the genetic engineering of these microorganisms. An important implication of this work is that localized temperature increases might lead to circumvention of restriction barriers and to more efficient exchange of genetic material between gram-negative and gram-positive bacteria under natural conditions.

This work was supported by BMFT grant 038409 in collaboration with Degussa AG.

LITERATURE CITED

1. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39**:440–452.

2. **Bailey, C. R., and J. D. Winstanley.** 1986. Inhibition of restriction in *Streptomyces clavuligerus* by heat treatment. J. Gen. Microbiol. **132:**2945–2947.
3. **Beringer, J. G.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
4. **Bertani, G., and J. J. Weigle.** 1953. Host controlled variation in bacterial viruses. J. Bacteriol. **65:**113–121.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
6. **Bousfield, I. J.** 1972. A taxonomic study of some coryneform bacteria. J. Gen. Microbiol. **71:**441–455.
7. **Buchanan-Wollaston, V., J. E. Passiatore, and F. Cannon.** 1987. The *mob*- and *ori*T mobilization functions of a bacterial plasmid promote its transfer to plants. Nature (London) **328:**172–175.
8. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
9. **Datta, N., and R. W. Hedges.** 1972. Host ranges of R factors. J. Gen. Microbiol. **70:**453–460.
10. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108:**1244–1249.
11. **Engel, H. W. B., N. Soedirman, J. A. Rost, W. J. van Leeuwen, and J. D. A. van Embden.** 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. J. Bacteriol. **142:**407–413.
12. **Engel, P.** 1987. Plasmid transformation of *Streptomyces tendae* after heat attenuation of restriction. Appl. Environ. Microbiol. **53:**1–3.
13. **Mazodier, P., R. Petter, and C. Thompson.** 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. **171:**3583–3585.
14. **Meselson, M., and R. Yuan.** 1968. DNA restriction enzyme from *E. coli*. Nature (London) **217:**1110–1114.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Miwa, K., K. Matsui, M. Terabe, S. Nakamori, K. Sano, and H. Momose.** 1985. Cryptic plasmids in glutamic acid-producing bacteria. Agric. Biol. Chem. **48:**2901–2903.
17. **Mojica-A, T., and R. B. Middleton.** 1971. Fertility of *Salmonella typhimurium* crosses with *Escherichia coli*. J. Bacteriol. **108:** 1161–1167.
18. **Olsen, R. H., and P. Shipley.** 1973. Host range and properties of the *Pseudomonas* R factor R1822. J. Bacteriol. **113:**772–780.
19. **Santamaria, R., J. A. Gil, J. M. Mesas, and J. F. Martin.** 1984. Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130:**2237–2246.
20. **Satta, G., J. G. Lorraine, and A. B. Pardee.** 1979. Degradation of *Escherichia coli* DNA: evidence for limitation *in vivo* by protein X, the *rec*A gene product. Mol. Gen. Genet. **168:**69–80.
21. **Seiler, H.** 1983. Identification key for coryneform bacteria derived by numerical taxonomic studies. J. Gen. Microbiol. **129:**1433–1471.
22. **Simon, R., M. O'Connell, M. Labes, and A. Pühler.** 1986. Plasmid vectors for the genetic analysis and manipulation of *Rhizobia* and other gram-negative bacteria. Methods Enzymol. **118:**640–659.
23. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1:**784–794.
24. **Thierbach, G., A. Schwarzer, and A. Pühler.** 1988. Transformation of spheroplasts and protoplasts of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **29:**356–362.
25. **Trieu-Cuot, P., C. Carlier, and P. Courvalin.** 1988. Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*. J. Bacteriol. **170:**4388–4391.
26. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
27. **Volkert, R. M., D. F. Spencer, and A. J. Clark.** 1979. Indirect and intergenic suppression of the *lex*A102 mutation in *E. coli* B/r. Mol. Gen. Genet. **177:**129–137.
28. **Willetts, N., and C. Crowther.** 1981. Mobilization of the nonconjugative *Inc*Q plasmid RSF1010. Genet. Res. **37:**311–316.