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Transfer of Tn*925* and Plasmids between *Bacillus subtilis* and Alkaliphilic *Bacillus firmus* OF4 during Tn*925*-Mediated Conjugation

ARTHUR A. GUFFANTI, PHILIP G. QUIRK, AND TERRY A. KRULWICH*

Department of Biochemistry, Mount Sinai School of Medicine of City University of New York, New York, New York 10029

Received 26 July 1990/Accepted 12 December 1990

Conjugative transposon Tn*925* was transferred to alkaliphilic *Bacillus firmus* OF4 during mating experiments, as monitored by the acquisition of tetracycline resistance at pH 7.5 and confirmed by Southern analysis of chromosomal DNA from transconjugants. Tetracycline resistance could not be demonstrated at pH 10.5, but transconjugants retained resistance upon growth at pH 7.5 after having grown for several generations at pH 10.5. When the *Bacillus subtilis* donor strain contained plasmids, either pUB110 or pTV1, in addition to Tn*925*, transfer of the plasmid to the alkaliphile occurred during conjugation, either together with or independently of the transfer of the transposon. The plasmids were stable in *B. firmus* OF4, expressing their resistance markers for kanamycin or chloramphenicol at pH 7.5 after growth of the transformants at high pH. Transconjugant *B. firmus* OF4, which carried Tn*925*, could serve as the donor in mating experiments with *B. subtilis* lacking the transposon. These studies establish a basis for initiation of genetic studies in this alkaliphilic *Bacillus* species, including the introduction of cloned genes and the use of transposon-mediated insertional mutagenesis.

Alkaliphilic *Bacillus* species are of both bioenergetic and biotechnological interest (10, 13). The most extensively studied species are obligate aerobes that grow optimally at pHs in the range of 10 to 11 on either sugars or nonfermentative carbon sources. Because various alkaliphiles produce extracellular enzymes that are stable under unusually alkaline conditions, efforts have been successfully mounted to express the genes encoding these potentially useful enzymes in *Escherichia coli* (10, 13). By contrast, the development of procedures for transfer of DNA to alkaliphiles, and hence viable genetic and molecular approaches to the physiology of these organisms, have lagged behind. There was one report of a lytic bacteriophage (11) and of an endogenous plasmid from one alkaliphilic strain (18). Recently, Kudo et al. (14) reported plasmid transformation of a facultative alkaliphile by a protoplast polyethylene glycol method. With our alkaliphilic strain, however, this precise protocol and several variations of it did not prove effective. With this modest background in mind, we recently undertook a substantial effort to develop molecular approaches to the facultatively alkaliphilic species *Bacillus firmus* OF4. The choice of this particular species was based on its ability to grow at nearly neutral as well as extremely alkaline pH (7), extensive available data on its major bioenergetic properties (13), the availability of mutant derivatives with at least one auxotrophic and/or antibiotic resistance marker (3), and successful experience in cloning the genes encoding the F_1F_0 ATPase from it and its obligately alkaliphilic homolog (12).

Initial efforts to establish gene transfer approaches to *B. firmus* OF4 included attempts to isolate bacteriophages from soil samples that had been enriched with the organism and attempts to achieve transformation with plasmids from *Bacillus subtilis* by electroporation. No bacteriophage have yet been isolated for *B. firmus* OF4. Electroporation of this strain with several different plasmids under a large variety of experimental conditions at nearly neutral pH resulted in only very low frequencies of transformation. Only with one plasmid, pUB110, were transformants observed in over one-third of apparently replicate experiments. Kanamycin-resistant transformants obtained from such experiments could be shown to express the resistance marker and harbor the plasmid after growth at pH 10.5; plasmids isolated from such transformants showed a restriction pattern identical to that of the initial pUB110 and actively transformed both *B. subtilis* and *B. firmus* OF4. However, the frequencies of transformation of the alkaliphile were still too low to be useful in the development of a gene transfer system, and no obvious vehicle for transposon mutagenesis emerged from these studies (8). Recently, therefore, we turned to a conjugative transposon that has mobilized its own transfer from *Enterococcus faecalis* to other gram-positive organisms and has facilitated plasmid transfer during transposon-mediated conjugation in species of *Bacillus* (2) and *Lactobacillus* (19). We here report that Tn*925*-mediated conjugation allows the transfer of the transposon from *B. subtilis* to alkaliphilic *B. firmus* OF4 and that plasmids can be delivered during this conjugation event either together with or independently of the transfer of Tn*925*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For experiments in which the alkaliphilic property of transconjugant strains was verified or in which the stability of genetic markers after growth at high pH was probed, facultatively alkaliphilic *B. firmus* OF4811M was grown at pH 10.5 on the D, L-malate-containing medium that we have routinely employed for the growth of alkaliphiles (9). *B. subtilis*, the donor strain in most experiments, cannot grow at pH 10.5. For the mating experiments, *B. firmus* OF4811M and the strains of *B. subtilis* were grown in MMB medium (2) adjusted to pH 7.5 with NaOH. Selective agar plates for determination of transposon or plasmid transfer to *B. firmus* OF4811M from *B. subtilis* contained the following medium:

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and transposons used in this study

Strain or plasmid	Genetic markers	Plasmid content	Reference or source
Bacteria			
B. firmus OF4811M	*met* Str^r	None	3
B. subtilis			
CU4050	*trpC2* Tet^r (Tn*925*)	None	S. Zahler
CU4353	*trpC2* Tet^r (Tn*925*)	None	S. Zahler
MS4060	*trpC2* Tet^r Kan^r (Tn*925*)	pUB110	This study
MS4071	*trpC2* Tet^r Cam^r (Tn*925*)	pTV1	This study
BD99	*trp thr his*	None	A. Garro
CU1065	*trpC2*	None	S. Zahler
MS1066	*trpC2*	pUB110	This study
MS1067	*trpC2*	pTV1	This study
Plasmid pCF10	Tet^r (Tn*925*)		G. Dunny

50 mM D,L-malate, 95 mM Na_2HPO_4, 5 mM KH_2PO_4, 0.1% (wt/vol) $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$, 0.05% Casamino Acids, 100 μg of L-methionine per ml, 0.1% trace salts (9), 0.02% yeast extract, and 1.5% agar. Antibiotics were added to the following final concentrations where indicated: 5 μg of chloramphenicol per ml, 10 μg of kanamycin per ml, and 2 μg of tetracycline per ml. On this medium, the donor strain formed only a light lawn because of the low levels of tryptophan, and the *B. firmus* OF4 transconjugants were discrete, yellowish colonies on that lawn. Once isolated as single colonies by streaking on pH 7.5 agar plates without antibiotics, the putative transconjugants of *B. firmus* OF4811M were checked for their ability to grow on antibiotics on pH 7.5 plates; this second screening employed plates with higher yeast extract content (0.1%), since suppression of the donor strain was no longer relevant, and lower concentrations of antibiotics (i.e., 0.1 μg of tetracycline per ml, 0.5 μg of chloramphenicol per ml, or 0.2 μg of kanamycin per ml). The wild-type *B. firmus* OF4811M was found to be sensitive to very low concentrations of antibiotics relative to *B. subtilis*, so that once the donor *B. subtilis* was no longer present, the concentrations of antibiotics used in the plating of the initial mating mix were too high to allow growth of the transconjugants. Presumably, during the initial plating of the mating mixture, the lawn of antibiotic-resistant donor lowered the effective concentration of antibiotic in the plates so that the transconjugants could grow. The lowered antibiotic concentrations in the second screening medium allowed growth of transconjugants of the alkaliphile, while still being considerably in excess of the concentration required to completely inhibit growth of the alkaliphile parent strain.

Preparation of plasmids, chromosomal DNA, and Southern analysis. Chromosomal DNA was isolated and purified by the method of Marmur (15), and plasmids were prepared by the method of Guerry et al. (6). For Southern analysis (17), pCF10, an *Enterococcus* plasmid carrying Tn*925* (1), was labeled by random primer extension (5) and used as the probe for hybridization (16) with *Hin*dIII-digested chromosomal DNA preparations from *B. subtilis* and *B. firmus* OF4 strains.

Transformation and conjugation procedures. For introduction of plasmids into the donor strain *B. subtilis* CU4050 or the isogenic strain CU1065 without Tn*925*, the strains were first grown to competence and then transformed with the desired plasmid as described by Dubnau and Davidoff-Abelson (4). Transfer of Tn*925* and/or plasmids between *B. subtilis* and *B. firmus* OF4811M was achieved by using the broth mating procedure of Christie et al. (2). Overnight cultures (2 ml) of donor and recipient were each diluted 1:1 with fresh MMB medium (pH 7.5). After incubation for 2 h at 30°C, 200 μl of donor suspension and 2 ml of recipient suspension were combined and incubated for another 4 h and then plated onto antibiotic selective medium.

RESULTS

Transfer of tetracycline resistance between *B. subtilis* and *B. firmus* OF4811M during conjugation. Upon mating with *B. subtilis* CU4050, tetracycline-resistant transconjugants of *B. firmus* OF4811M were observed. The identity of the transconjugants was confirmed by growth of these strains at pH 10.5 (at which tetracycline resistance is not demonstrable) and their retention of the resistance marker upon replating at pH 7.5. Use of the *B. firmus* OF4811M strain made it possible to additionally check the two markers carried by that strain, so that the identity of the transconjugants could also be confirmed without plating at pH 10.5. This would be important in experiments in which transpositions that lead to loss of the capacity for alkaliphily were sought. The *B. firmus* OF4811M transconjugants still required methionine. The frequency of appearance of tetracycline-resistant transconjugants was unaffected by inclusion of 50 μg of DNase per ml in the mating mixture. Neither the mixture filtrate nor the donor that did not harbor Tn*925* could substitute for the *B. subtilis* donor that contained the conjugative transposon. When one of the tetracycline-resistant transconjugants was used as the donor in a mating experiment, with a strain of *B. subtilis* lacking the transposon used as the recipient, tetracycline-resistant transconjugants of *B. subtilis* arose that still required tryptophan. It thus appeared that Tn*925*-mediated conjugation between the two *Bacillus* species could occur in either direction.

The tetracycline resistance marker was transferred to *B. firmus* OF4811M from either CU4050 or a transformant thereof, MS4071, that also contained pTV1 at a frequency of approximately 6.5×10^{-7} per donor and 3.5×10^{-7} per recipient. The equivalent values for strain CU4050 were 5.6×10^{-7} and 2.7×10^{-7}, respectively. To verify that transfer of the resistance marker represented transfer to and transposition in the recipient by Tn*925*, chromosomal DNA from two transconjugants of *B. firmus* OF4811M was subjected to Southern analysis with the Tn*925*-containing plasmid pCF10 as the probe. The plasmid itself, cut with *Hin*dIII, exhibited

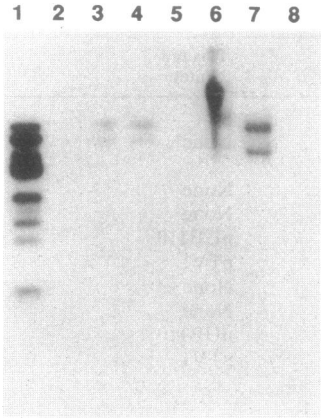

FIG. 1. Southern transfer analysis of chromosomal DNA of transconjugants of *B. firmus* OF4811M. Radiolabeled pCF10 was used to probe blots from an electrophoretic separation of fragments from *Hin*dIII digests of the following samples (lanes): 1, pCF10; 2, *B. subtilis* BD99; 3 and 4, *B. subtilis* CU4050 grown with and without tetracycline, respectively; 5, *B. firmus* OF4811M; 6, *B. firmus* OF4811M transconjugant from a mating with *B. subtilis* CU4353; 7, *B. firmus* OF4811M transconjugant from a mating with *B. subtilis* CU4050; 8, lambda standards.

the pattern expected on the basis of work in other laboratories (2), and similarly digested chromosomal DNA from the Tn*925*-containing *B. subtilis* donor strain and the two transconjugant *B. firmus* OF4811M strains exhibited hybridization with the probe (Fig. 1). The chromosomal DNA of each strain showed the two fragments expected from the digestion of the Tet^r element of pCF10, which has a single site for *Hin*dIII. Control DNA preparations from strains of both *Bacillus* species that lacked the transposon were negative.

Transfer of plasmids to *B. firmus* OF4 during Tn*925*-mediated conjugation. Mating of *B. firmus* OF4811M with derivatives of CU4050 that had been transformed with either pUB110 (MS4060) or pTV1 (MS4071) resulted in the appearance of some transconjugants that acquired the resistance marker of the plasmid. In control experiments, in which *B. firmus* OF4811M was mated with MS1066 or MS1067 containing pUB110 or pTV1, respectively, but no Tn*925*, no resistance to the plasmid marker was found in the alkaliphile. When appropriate selections and rescreenings were conducted after the mating, it was possible to discern the approximate proportion of transconjugants in matings that acquired Tn*925* alone, Tn*925* and the plasmid, and the plasmid alone. Transconjugants that acquired the resistance marker of the Tn*925* alone and those that acquired both that marker and the resistance marker of the plasmid were represented in approximately equal proportions (Table 2). Transconjugants that acquired only the plasmid occurred at a frequency that was roughly 10% of those other two categories. In some earlier matings between *B. firmus* OF4811M and *B. subtilis* CU4353, we were able to detect resistance to lincomycin, presumably encoded by the Tn*917* part of the composite Tn*925*:Tn*917* harbored by that particular donor strain. In subsequent experiments with CU4353 and with the pTV1-containing transconjugants, we were unable to clearly and reproducibly demonstrate lincomycin or erythromycin resistance. On the other hand, the kanamycin and chloramphenicol markers were reproducibly detected in the *B. firmus* OF4811M on plates at pH 7.5 both before and after streaking onto plates at pH 10.5.

TABLE 2. Transfer of plasmids during Tn*925*-mediated conjugation

B. subtilis donor	No. of transconjugants per *B. firmus* OF4811M recipient				
	Tet^r	Cam^r	Cam^r Tet^r	Kan^r	Kan^r Tet^r
MS4071[a]	1.8×10^{-7}	1.5×10^{-8}	1.7×10^{-7}		
MS4060[b]	9×10^{-8}			1×10^{-8}	7×10^{-8}

[a] The first selection was for Tet^r or Cam^r. At least 100 colonies of each type were then tested for resistance to the other antibiotic marker to yield Cam^r Tet^r frequency. The frequency of resistance to only one marker (Tet^r or Cam^r) was calculated from the number of original Tet^r or Cam^r colonies that were not resistant to the other antibiotic.

[b] The first selection was for Tet^r or Kan^r, and frequencies of Tet^r alone, Kan^r alone, and Tet^r and Kan^r together were calculated as for strain MS4071.

DISCUSSION

The results presented here demonstrate that the conjugative transposon Tn*925* is capable of mobilizing its own transfer between *B. subtilis* and *B. firmus* OF4. The transposon is integrated into the alkaliphile recipient chromosome, where it is stable and capable of conferring tetracycline resistance at pH 7.5 even after growth of the transconjugants at pH 10.5. During the mating event, recipient alkaliphile cells can also acquire plasmids contained in the donor strain. Since approximately 10% of the transconjugants in such a mating receive the plasmid without the conjugative transposon, the transfer of the plasmid does not appear to depend upon any intermediate form involving the transposon. Plasmid transfer is, however, clearly dependent upon the conjugative transposon Tn*925*, as has been observed for transfer of plasmids from one *B. subtilis* strain to another during conjugation (20).

Although the frequencies of conjugal transfer of Tn*925* and/or plasmids in these experiments were not extremely high, they were sufficient to offer a viable option for introduction of useful transposons and cloning vectors into the alkaliphile. The exquisite sensitivity of *B. firmus* OF4 strains to at least some antibiotics and the difficulty in achieving reproducible expression of some resistant markers are notable but should not impede progress toward genetic manipulation of this organism with the general approach developed here.

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