JOURNAL OF BACTERIOLOGY, May 1995, p. 2583–2588
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 9

Genetic Transfer by Conjugation in the Thermophilic Green Sulfur Bacterium *Chlorobium tepidum*

THOMAS M. WAHLUND† AND MICHAEL T. MADIGAN*

Department of Microbiology, Southern Illinois University, Carbondale, Illinois 62901-6508

Received 12 December 1994/Accepted 28 February 1995

The broad-host-range IncQ group plasmids pDSK519 and pGSS33 were transferred by conjugation from *Escherichia coli* into the thermophilic green sulfur bacterium *Chlorobium tepidum*. *C. tepidum* exconjugants expressed the kanamycin and ampicillin-chloramphenicol resistances encoded by pDSK519 and pGSS33, respectively. Ampicillin resistance was a particularly good marker for selection in *C. tepidum*. Both pDSK519 and pGSS33 were stably maintained in *C. tepidum* at temperatures below 42°C and could be transferred between *C. tepidum* and *E. coli* without modifications. Conjugation frequencies ranged from 10^{-1} to 10^{-4} exconjugants per donor cell, and frequencies of 10^{-2} to 10^{-3} were consistently obtained when ampicillin resistance was used as a selectable marker. Methods for growth of *C. tepidum* on agar, isolation of plating strains and antibiotic-resistant mutants of wild-type *C. tepidum* cells, and optimum conditions for conjugation were also investigated.

The recently isolated anoxygenic phototrophic bacterium *Chlorobium tepidum* is the only known thermophilic member of the family *Chlorobiaceae* (green sulfur bacteria) (27). In addition to its thermophilic phenotype, the growth rate of *C. tepidum* cultured under photoautotrophic conditions (generation time ~2 h) is faster than that of any other anoxygenic phototroph, making it an ideal research tool for basic studies of photosynthesis and autotrophy in green bacteria. *C. tepidum* is also unique in its ability to fix N_2 up to 60°C, with optimum nitrogenase activities occurring at the organism's growth temperature optimum of 48°C (26).

To date, most research on fundamental biological processes such as photosynthesis, N_2 fixation, and CO_2 fixation in anoxygenic phototrophic bacteria has focused on nonsulfur purple bacteria, especially species of the genus *Rhodobacter*. This is largely due to their ease of culture (16) and the availability of efficient gene transfer systems (28). Although genetic transfer systems have been lacking in green bacteria, molecular biological approaches have been employed to identify and isolate several genes from these organisms (4, 5, 23, 30). In this connection, genes coding for two key proteins in the chlorosome, the unique antenna system of green bacteria (5), have been cloned and sequenced, as has an operon encoding reaction center genes (4). Now that green bacteria have entered the era of molecular biology, genetic tools are needed to study the expression and regulation of these genes and their products. In this report, we describe the development of an effective method for conjugation of *C. tepidum* with two IncQ-based broad-host-range plasmids. We also describe optimal conditions for conjugation and techniques for efficient plating and manipulation of *C. tepidum*, which are necessary for classical genetic analyses of photosynthesis in green bacteria.

Bacterial strains and plasmids. The organisms and plasmids used in this study are listed in Table 1. *C. tepidum* TLS was described previously (27), as was *C. limicola* (*vibrioforme*) forma *thiosulfatophilum NCIB 8327 (26).*

Culture media. *Chlorobium* species were maintained in liquid medium Pf-7 as previously described (26, 27). A new medium designed for growth of *C. tepidum* on agar plates, designated CP (chlorobium plating) medium, was prepared by addition of the following components to 900 ml of deionized water: concentrated salts (distilled H_2O, 967 ml; EDTA, 500 mg; $MgSO_4 \cdot 7H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$, 2.5 g; NaCl, 20 g), 20 ml; KH_2PO_4, 0.5 g; ammonium acetate, 0.5 g; NH_4Cl, 0.4 g; $Na_2S_2O_3 \cdot 5H_2O$, 2.3 g; trace elements (27), 1 ml; vitamin B_{12}, 20 μg; cysteine, 0.36 g; MOPS (3-*N*-morpholinopropanesulfonic acid), 2.1 g. The pH was adjusted to 7.20 by slow addition of 10 N NaOH. The volume was brought to 1 liter, and agar (Difco) was added to a 1.5% final concentration. Following autoclaving, the medium was cooled to 55 to 60°C, dispensed into petri dishes under an evaporative hood, and allowed to cool for approximately 20 min. Upon cooling, the pH of this medium dropped to between 6.9 to 7.0, within the narrow pH range for optimum growth of *C. tepidum* (27). The plates were then immediately placed into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under an atmosphere of N_2-H_2 (98:2). Alternatively, the plates could be stored in an anaerobic jar with a GasPak envelope (H_2-CO_2 generator) prior to use. However, it was found essential for pH stability that this plating medium be kept anaerobic.

For diazotrophic growth of *C. tepidum* on agar, the protocol for preparation of CP medium was modified as follows: sodium acetate (0.53 g) was substituted for ammonium acetate, and NH_4Cl and cysteine were omitted. The pH of this medium, designated, CPNF, was adjusted to 6.9 to 7.0 prior to autoclaving, but unlike CP medium, no decrease in pH was observed following cooling to room temperature. CPNF medium supported good growth of *C. tepidum* on N_2, provided that the medium was kept anaerobic. Media for growth of *C. tepidum* on N_2 in liquid culture and growth measurement in liquid culture were described previously (26).

CP medium supplemented with 0.01% yeast extract was employed in conjugations of *C. tepidum* with *Escherichia coli* strains and was designated CPC medium. Liquid CP medium and CPNF were prepared as described above and then dispensed into 17-ml screw-cap tubes or 250-ml bottles and stored in an anaerobic chamber with the caps loose for 1 to 2 days to ensure anaerobiosis. All of the media discussed above also

* Corresponding author. Electronic mail address: madigan@qm.c-micro.siu.edu.

† Present address: Department of Microbiology, The Ohio State University, Columbus, OH 43210-1292.

TABLE 1. Organisms and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Bacterial strains		
Escherichia coli		
HB101	λ$^-$ *recA13 proA2 leu lacY1 galK2 xyl-5 mtl-1 ara-14* F$^-$ *hsdS20* (r_B^- m_B^-) *supE44 rpsL20*	3
S17-1	*pro thi hsdR17* (r_K^- m_K^+) *recA* RP4-2(Tc^r::Mu-Km^r::Tn*7*)	25
DH5	φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* F$^-$ Δ(*lacZYA-argF*)*U169*	8
Chlorobium tepidum		
TLS-1	Wild type	27
WT2321	Plating strain derivative of strain TLS-1	This study
Nal-1	Derivative of strain TLS-1; nalidixic acid resistant	This study
Plasmids		
pDSK519	IncQ; Km^r	12; J. Wall
pKT230	IncQ; Km^r Sm^r	2
pGSS33	IncQ; Ap^r Cm^r Tc^r	24
pRK290	IncP; Tc^r	6
pRK415	IncP; Tc^r	12
pGM415	pRK415 derivative; Gm^r	15
pVK100	IncP; Tc^r Km^r; cosmid	D. Clark
pURF047	IncW; Mob$^+$ Par$^+$ Gm^r	J. Wall
pRK2013	Helper plasmid; Km^r	7
pRK2073	pRK2013::Tn*7* helper plasmid; Sp^r Tp^r	J. Wall

supported growth of the mesophilic species *C. limicola* (*vibrioforme*) forma *thiosulfatophilum* NCIB 8327.

Growth on solid media. Growth of *C. tepidum* on agar plates required anaerobic conditions and a mechanism for sulfide generation within the anaerobic jar. The procedure used was a modification of that originally devised by Irgens (11). The method required a GasPak Plus (BBL) envelope (H_2-CO_2 generator), 0.1 to 0.05 g of thioacetamide (TAA; Sigma Chemical Co. [St. Louis, Mo.]), an Oxoid anaerobic jar containing the low-temperature Oxoid catalyst system (Unipath, Ogdensburg, N.Y.), and 0.2 to 0.5 N HCl. The protocol was as follows. *C. tepidum* cells were plated in the anaerobic chamber and placed into the anaerobic jar. A small test tube containing 0.1 g of TAA was secured to the inside of the jar at the base with tape. The GasPak Plus envelope was activated, 1 ml of 0.2 to 0.5 N HCl was added to the test tube containing TAA, and the jar was sealed. The jar was left within the anaerobic hood until the pressure gauge (contained within the lid of the jar) indicated that there were no leaks in the system. The jar was then taken out of the anaerobic chamber and incubated in an illuminated water bath at 47 to 48°C (37 to 39°C for conjugation experiments and also for growth of *C. limicola* 8327). Single colonies of *C. tepidum* were visible within 3 to 4 days (lawns appeared overnight). Although the evolution of sulfide within the anaerobic jar eventually poisons the palladium catalyst, it was found that Oxoid catalysts could be regenerated in a drying oven (~80°C overnight) and used several times without loss of catalytic activity.

E. coli strains were grown aerobically in LB medium (22) at 37°C, and the appropriate antibiotics were employed at the following concentrations (in micrograms per milliliter): ampicillin (AP), 100; kanamycin (KM), 25; chloramphenicol (CM), 20; streptomycin, 50; spectinomycin, 100. The antibiotic concentrations used for *Chlorobium* strains are discussed below.

Conjugation. The recipient strains used in all conjugation experiments were plating strain derivatives (see below and Table 1) of *C. tepidum* TLS. In most conjugation experiments, broad-host-range plasmids were mobilized by using helper plasmid pRK2073 (Table 1). As an alternative to triparental matings, plasmids were mobilized in biparental conjugation experiments with *E. coli* S17-1 as the donor (Table 1).

For triparental matings, *C. tepidum* recipient strains were grown overnight in medium Pf-7 to the late exponential or early stationary phase (~800 to 900 Klett units; no. 66 filter) and then taken into the anaerobic chamber for further manipulations. Overnight cultures of *E. coli* were diluted 10^{-1} in LB (without antibiotic) and incubated at 37°C with shaking for 2 h. Donor and helper cells (1 ml) were then washed, resuspended in LB, and placed in the anaerobic chamber for mating. Matings were set up by combining recipient cells (0.4 ml) with donor and helper cells (0.3 ml of each) in a 1.5-ml Eppendorf tube and centrifuging them inside the chamber for 4 min at 13,600 × *g* in a microcentrifuge (Nal-1 recipient cells were washed three times in CP medium prior to mating to remove nalidixic acid). It should be noted that centrifugation of *C. tepidum* cells could also be done outside the anaerobic chamber without affecting conjugation, provided that cells were returned before removing the supernatant. The mating mixture pellet was resuspended in 200 ml of CP medium (supplemented with 0.05% yeast extract), and four 50-μl aliquots were spotted onto a well-dried CPC medium plate. Control plates containing either recipient cells (only) or recipient cells plus donor (no helper) cells were prepared as described above and included in each conjugation experiment. All three CPC plates were placed in an anaerobic jar and incubated in ca. 5,000 lx of incandescent light at 37 to 40°C in a water bath for various amounts of time. The sulfide-generating system (see above) was not employed during matings.

Following mating, jars were taken inside the anaerobic chamber and the cells from each plate were resuspended with 1 to 1.5 ml of CP medium. Dilutions of 10^{-1} to 10^{-4} were plated on CP medium plates containing the appropriate antibiotic(s). In some experiments, when *C. tepidum* Nal-1 (Table 1) was used as the recipient, 0.2 ml of CP medium containing nalidixic acid (20 μg/ml) was spread on the mating plates and incubation was continued for 3 to 4 h to allow expression of the

antibiotic resistance marker on the plasmid. Selection was done in all experiments by incubation in the light at 37 to 40°C with the sulfide-generating system (described above) until colonies appeared (3 to 6 days). Counterselection, in most cases, was by prototrophy of *C. tepidum* (Table 1) and also, in some cases, by the nalidixic acid sensitivity of the donor and helper. Conjugation frequencies were calculated as the number of exconjugants per donor cell. Viable counts of recipient cells on each CPC plate were done to ensure that less than one doubling of recipient cells had occurred during the mating time period.

Other methods and chemicals. Plasmid DNA isolation and transformation of *E. coli* were performed as previously described (9, 22). *C. tepidum* exconjugant colonies from selection plates were grown overnight in liquid medium Pf-7 (27) containing the appropriate antibiotic and checked for purity by streaking on LB plates containing the antibiotic resistance marker of the *E. coli* donor and helper. Plasmid DNA was isolated from *C. tepidum* exconjugants by the alkaline lysis method (22) with one minor modification: swirling the cells in a water bath (45 to 50°C) during the sodium dodecyl sulfate lysis step improved cell lysis and recovery of plasmid DNA from *C. tepidum*. All chemicals were of reagent grade, and most (including thiosulfate and TAA) were obtained from Sigma Chemical Co.

Growth of *C. tepidum* on plates. Before approaching the problem of genetic transfer in *C. tepidum*, it was necessary to be able to grow this organism on solid media. Initial attempts to grow *C. tepidum* on plates employing media and conditions described for growth of the mesophilic species *C. limicola* forma *thiosulfatophilum* 8327 (21) failed, necessitating the development of a new plating medium containing diffusable sulfide (H_2S) in the headspace instead of soluble sulfide (HS^-) in the agar medium (CP medium; see above). Once conditions that would allow plating of *C. tepidum* were established, it was observed that cell numbers calculated from plating experiments were several logs lower than corresponding microscopic counts. However, efficient "plating strain" derivatives of *C. tepidum* could be obtained by picking large colonies that did come up, growing them overnight in liquid medium, and then replating them on CP medium; such strains demonstrated plating efficiencies ranging from 90 to 100% of the microscopic count. *C. tepidum* WT2321, used in this study, plated out at nearly 100% efficiency and produced the largest colonies of any of the plating isolates examined (Fig. 1). Plating strain derivatives of *C. limicola* forma *thiosulfatophilum* 8327 were obtained in a similar way. Other phenotypic properties of plating strain derivatives and the original unplated cultures of *C. tepidum* seemed to be the same.

Antibiotic resistance and generation of useful selectable markers in *C. tepidum*. Because one of the primary factors involved in the development of new genetic systems is the availability of useful selectable markers, we evaluated the sensitivity of *C. tepidum* to various antibiotics. Several *C. tepidum* spontaneous mutant derivatives of strain WT2321 resistant to a given antibiotic were isolated by plating mid-exponential to early stationary phase cells on CP medium plates containing increasing levels of a particular antibiotic. The antibiotic resistance of each mutant was confirmed by growing resistant colonies overnight in liquid culture containing the appropriate antibiotic and then by plating cells on CP medium plates containing the highest antibiotic concentration which would support growth in liquid culture. Initial results of these experiments showed strain WT2321 to be somewhat resistant to aminoglycosides at 48°C and that resistance was dose dependent (Table 2). Moreover, the effectiveness of aminoglycosides against *C. tepidum* decreased significantly when the incubation temperature was lowered to 37°C, the temperature to be used for conjugations with *E. coli* (see below); apparent natural resistance in *C. tepidum* increased by 2 to 3 orders of magnitude when cells were incubated at 37 versus 48°C (Table 2). However, three antibiotics remained highly effective against *C. tepidum* at both 37 and 48°C (AP, CM, and tetracycline), resistances to two of which (AP and CM) were useful as selectable markers in *C. tepidum.*

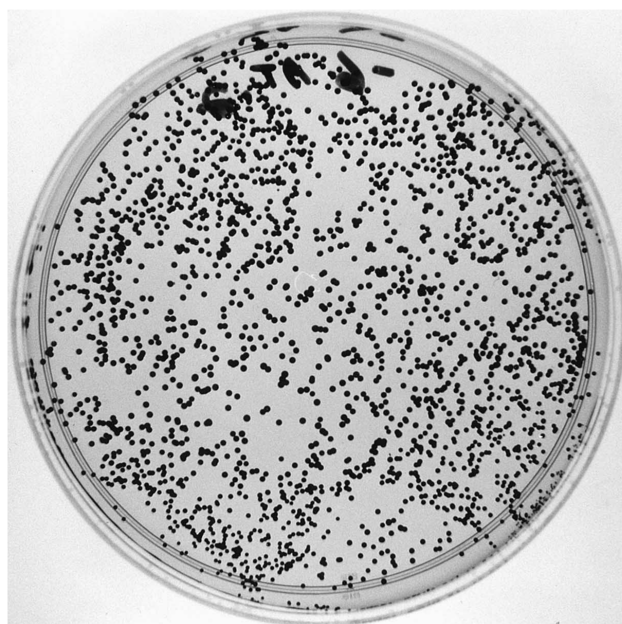

FIG. 1. Colonies of *C. tepidum* WT2321 on a CP medium plate. An overnight culture (ca. 950 photometer units) was diluted by 10^{-6}, and 0.1 ml was plated on a CP medium plate and incubated photosynthetically at 48°C in the TAA jar system. The viable count calculated from this plate was 6×10^9 cells · ml^{-1}.

Conjugal transfer of IncQ group plasmids into *C. tepidum*. Despite some background problems with the use of aminoglycosides as selectable markers in *C. tepidum* (see above), initial conjugation experiments with *E. coli* and *C. tepidum* were carried out with IncQ group plasmid pDSK519 (which contains Km^r as a selectable marker) and the results demonstrated successful transfer of the plasmid into *C. tepidum*; conjugations

TABLE 2. Effect of temperature on antibiotic resistance of *C. tepidum* WT2321

Antibiotic	Concn (μg/ml)	Apparent spontaneous mutation frequency[a] at:	
		48°C	37°C
KM	25	1.1×10^{-7}	4.5×10^{-4}
	50	2.1×10^{-8}	1.2×10^{-5}
	100	1.0×10^{-8}	1.0×10^{-5}
Streptomycin	25	8.2×10^{-7}	5.0×10^{-4}
	50	3.1×10^{-7}	7.5×10^{-5}
	200	$<10^{-10}$[b]	2.2×10^{-8}
AP	1	$<10^{-10}$	$<10^{-10}$
CM	10	$<10^{-10}$	$<10^{-10}$
Tetracycline	1	$<10^{-10}$	$<10^{-10}$

[a] Frequency is expressed as the number of antibiotic-resistant cells per viable cell at the temperature and antibiotic concentration specified.
[b] This value represents the limit of detection of spontaneous antibiotic-resistant mutants.

TABLE 3. Conjugation of *C. tepidum* with *E. coli* containing broad-host-range IncQ group plasmids

Recipient strain	Plasmid[a]	Selected marker (drug concn [μg/ml])	Mating time (h)	Conjugation frequency range[b]
WT2321	pDSK519	Km^r (25)	1.5	10^{-2}–10^{-3} (2)
		Km^r (25)	4.5	10^{-2}–10^{-3} (3)
		Km^r (50)	16–20	10^{-1}–10^{-2} (2)
	pKT230	Sm^r (50)	5–8	10^{-5}–10^{-6} (2)
	pGSS33	Ap^r (3)	1.5	10^{-2}–10^{-4} (2)
		Ap^r (3)	5	10^{-2}–10^{-3} (5)
		Ap^r (3)	16–20	10^{-2}–10^{-3} (10)
		Cm^r (5)	16–20	10^{-3}–10^{-4} (3)
		Cm^r (20)	16–20	$<10^{-10}$–10^{-7} (3)[c]
		Tc^r (1)	20	$<10^{-10}$ (2)[c]
Nal-1	pGSS33	Ap^r (3)	1.5	10^{-2}–10^{-4} (2)
		Ap^r (3)	18	10^{-3}–10^{-4} (2)

[a] Matings with plasmid pDSK519 (KM at 25 μg/ml) were done at 40°C, and selection was done at 42°C. For all other conjugations described, both mating and selection were done at 37°C.

[b] Frequency is the number of exconjugants per donor cell. The number in parentheses is the number of experiments performed.

[c] 10^{-10} exconjugants per donor cell represents the limit of detection of this assay.

employing KM at 25 or, especially, 50 μg/ml produced transfer frequencies of pDSK519 at 37°C that were readily detectable (Table 3). No exconjugants were obtained when the KM concentration was increased to 100 μg/ml (data not shown). Restriction enzyme analysis of plasmid DNA extracted from a Km^r *C. tepidum* colony and electrophoresed against $CsCl_2$-purified pDSK519 from *E. coli* clearly showed the presence of this plasmid in *C. tepidum* (Fig. 2A). Confirmation of successful conjugation was obtained by transforming pDSK519 isolated from *C. tepidum* exconjugants into *E. coli* HB101 and reisolating the intact plasmid (data not shown).

The exquisite sensitivity of *C. tepidum* to AP, CM, and tetracycline at 37°C (Table 2) indicated that conjugation with IncQ plasmid pGSS33 (Table 1) might be even more successful. This versatile vector, which has been successfully introduced into the sulfate-reducing bacterium *Desulfovibrio desulfuricans* (1), centers resistance to each of these antibiotics and was used to evaluate plasmid transfer and optimal conditions for conjugation in *C. tepidum*. Table 3 shows typical results obtained in a conjugation involving pGSS33. In each of these experiments, development of antibiotic-resistant colonies was strictly dependent upon the presence of both *E. coli* donor and helper cells during mating; spontaneous background resistance was not a problem. Verification of transfer and stabilization of pGSS33 in *C. tepidum* were obtained by gel electrophoresis of plasmid preparations from *C. tepidum* WT2321 recipient cells, a WT2321 exconjugant, and an *E. coli*(pGSS33) donor (Fig. 2B). Plasmid DNA isolated from a *C. tepidum* exconjugant could be transformed back into *E. coli* and reisolated intact (Fig. 2B), thus providing additional confirmation of successful transfer of pGSS33 into *C. tepidum.*

Determination of optimal conditions for conjugation in *C. tepidum*. Several parameters relating to conjugation of *C. tepidum* with pGSS33 were evaluated. Mid-exponential to stationary phase cultures yielded similar conjugation frequencies

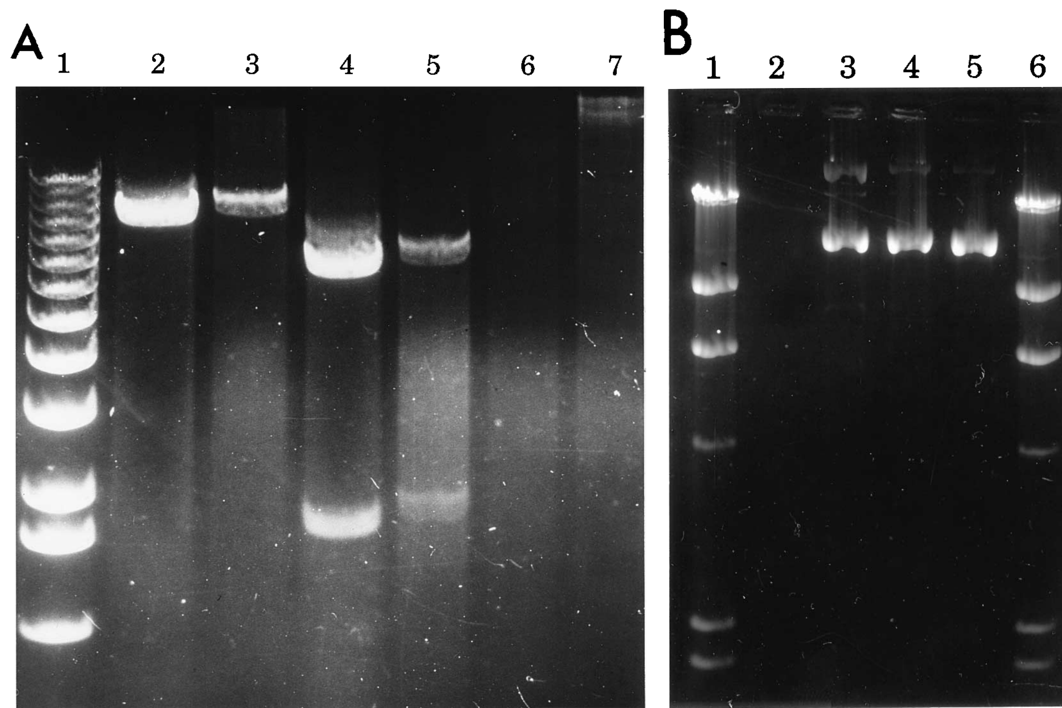

FIG. 2. Transfer of IncQ plasmids into *C. tepidum* WT2321. (A) Restriction analysis of plasmid preparations from a *C. tepidum* exconjugant digested with *Eco*RI (lane 2) and *Pvu*II (lane 4) electrophoresed against $CsCl_2$-purified pDSK519 plasmid DNA from *E. coli* digested with *Eco*RI (lane 3) and *Pvu*II (lane 5). Identical preparations from wild-type *C. tepidum* cells digested with *Eco*RI (lane 6) and *Pvu*II (lane 7) were run as controls. Lane 1 contained a 1-kb DNA ladder. *Eco*RI digestion of pDSK519 yielded a single fragment of 8.1 kb; *Pvu*II digestion yielded fragments of 6.5 and 1.7 kb. (B) Transfer of pGSS33 into WT2321 confirmed by restriction analysis of plasmid DNA preparations from the WT2321 recipient (lane 2), a WT2321 exconjugant (lane 3), an *E. coli* donor (lane 4), and pGSS33 DNA isolated from *C. tepidum* transformed back into *E. coli* cells and extracted (lane 5). pGSS33 DNA was digested with *Eco*RI, and the size (ca. 13.4 kb) was determined by comparison to *Hin*dIII fragments of phage lambda DNA (lanes 1 and 6). The molecular size standards (top to bottom) were 23, 9.4, 6.7, 4.4, 2.2, and 2.1 kb.

(data not shown). The minimum time required for transfer of pGSS33 was determined by using *C. tepidum* Nal-1 (Table 1) as the recipient; DNA transfer from *E. coli* cells was stopped at different times by addition of nalidixic acid. Transfer of pGSS33 from *E. coli* to *C. tepidum* could be detected within 90 min of mating (Table 3), although conjugations were not consistently successful in matings this short. However, because of the oxygen sensitivity of *C. tepidum* (27) and the numerous manipulations requiring use of the anaerobic chamber, conjugation times that allowed both transfer and expression prior to plating for selection were chosen. Therefore, we routinely performed conjugations overnight (16 to 20 h) even though comparable conjugation frequencies were obtained within 4 to 6 h of mating (Table 3). For overnight conjugations, the number of generations elapsed was determined from viable cell counts taken before and after conjugation, and transfer frequencies were normalized to give an accurate estimate of exconjugants.

The highest conjugation frequencies in experiments involving transfer of pGSS33 to strain WT2321 were observed when Ap^r (3 μg/ml) was used as the selectable marker (Table 3). A 4- to 15-fold decrease in transfer frequency was observed when exconjugants were selected with CM (5 μg/ml) instead of AP (Table 3). CM levels higher than 10 μg/ml reduced the number of exconjugants dramatically (Table 3). For unknown reasons, no evidence of conjugal transfer was detected with Tc^r (1 μg/ml) as the selectable marker, suggesting that Tc^r determinants were either not expressed or not stable in *C. tepidum* (this was further supported by the finding that Ap^r cells of *C. tepidum* remained Tc^s). Interestingly, Ap^r exconjugants selected at 3 μg/ml often tolerated only low levels of AP (1 μg/ml) for initial growth in liquid media. The reasons for this were unclear; however, once these exconjugants grew at low AP levels in liquid media, full cell densities were obtained overnight at concentrations as high as 10 to 15 μg of AP per ml. Conjugation of pGSS33 with *C. tepidum* Nal-1 as the recipient yielded conjugation frequencies similar to those observed with strain WT2321 (Table 3). Checks for *E. coli* contamination during manipulation of exconjugants were negative in all cases.

Transfer into *C. tepidum* of another IncQ plasmid, pKT230, was detected by using streptomycin at 50 μg/ml, although at a frequency not much above spontaneous background levels (compare data in Tables 2 and 3). Transfer of IncW plasmid pURF047 or IncP plasmid pGM415 could not be detected because of a high natural resistance of *C. tepidum* to gentamicin (data not shown). Results obtained with other IncP plasmids and Tc^r as the selectable marker were also negative, as were experiments involving IncP cosmid pVK100 (KM at 50 μg/ml).

Conclusions. This work has established a conjugation system for genetic studies of *C. tepidum*. Natural transformation of green sulfur bacteria has been demonstrated; however, these experiments involved either transfer of endogenous plasmids (17) or uptake and expression of chromosomal DNA (21) isolated from antibiotic-resistant mutants of the same strain; DNA from different strains or species was not transformable (21). Moreover, transformation efficiency is low and the process is inconsistent (13). However, electroporation of mesophilic chlorobia has also been recently demonstrated and apparently allows transfer of foreign DNA (13).

A limitation of our new genetic system involves the apparent instability of pGSS33 and pDSK519 at 48°C, preventing analysis of cloned gene products at the optimum growth temperature of *C. tepidum*. However, *C. tepidum* grows reasonably well at 37 to 40°C and the ability to do genetic studies directly with *C. tepidum* is preferable to the use of surrogate genetic techniques. Work is in progress towards construction of a thermostable shuttle vector for use in *C. tepidum*. Preliminary analysis of *C. tepidum* has not revealed an endogenous plasmid(s) which might be useful for vector construction, although it should be possible to construct thermostable shuttle vectors by using existing broad-host-range plasmids and replicons from plasmids isolated from other thermophiles (10, 29).

The focus of our work on green bacterial genetics has been *C. tepidum*. However, because of the close phylogenetic relationship between *C. tepidum* and nonthermophilic chlorobia (27), conjugation should occur in mesophilic *Chlorobium* species as well, and results of a single conjugation experiment with plasmid pGSS33 and *C. limicola* (*vibrioforme*) 8327 support this contention (data not shown). Nevertheless, because of its rapid growth characteristics, we predict that *C. tepidum* will be the organism of choice for further study of the molecular biology and genetics of green bacteria. The ability to transfer plasmid DNA into *C. tepidum* via conjugation as demonstrated herein should allow genetic analysis of the extensive biochemical and biophysical research already being done on this organism (14, 18–20, 26, 31).

This work was supported in part by grant 91-37305-6600 from the U.S. Department of Agriculture Competitive Grants Office and by a Dissertation Research Award (to T.M.W.) from Southern Illinois University.

We thank Judy Wall for providing a number of the plasmids used in this study.

REFERENCES

1. **Argyle, J. L., B. J. Rapp-Giles, and J. D. Wall.** 1992. Plasmid transfer by conjugation in *Desulfovibrio desulfuricans*. FEMS Microbiol. Lett. **94:**255–262.
2. **Bagdasarian, M., R. Lurz, B. Rückert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host-range, high copy number, RSF1010-derived vectors and a host vector system for gene cloning in *Pseudomonas*. Gene **16:**237–247.
3. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68:**245–267.
4. **Büttner, M., D.-L. Xie, H. Nelson, W. Pinther, G. Hauska, and N. Nelson.** 1992. Photosynthetic reaction center genes in green sulfur bacteria and in photosystem 1 are related. Proc. Natl. Acad. Sci. USA **89:**8135–8139.
5. **Chung, S., G. Frank, H. Zuber, and D. A. Bryant.** 1994. Genes encoding the chlorosome components from the green sulfur bacteria *Chlorobium vibrioforme* strain 8327D and *Chlorobium tepidum*. Photosynth. Res. **41:**261–275.
6. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host-range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
7. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
8. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557.
9. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
10. **Imanaka, T., M. Fujii, I. Aramori, and S. Aiba.** 1982. Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **149:**824–830.
11. **Irgens, R. L.** 1983. Thioacetamide as a source of hydrogen sulfide for colony growth of purple sulfur bacteria. Curr. Microbiol. **8:**183–186.
12. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
13. **Kjaerulff, S., D. B. Diep, J. S. Okkels, H. V. Scheller, and J. G. Ormerod.** 1994. Highly efficient integration of foreign DNA into the genome of the green sulfur bacterium, *Chlorobium vibrioforme*, by homologous recombination. Photosynth. Res. **41:**277–283.
14. **Kusumoto, N., K. Inoue, H. Nasu, and H. Sakurai.** 1994. Preparation of a photoactive reaction center complex containing photoreducible Fe-S centers and photooxidizable cytochrome *c* from the green sulfur bacterium *Chlorobium tepidum*. Plant Cell Physiol. **35**(1)**:**17–25.
15. **Labes, M., A. Pühler, and R. Simon.** 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. Gene **89:**37–46.
16. **Madigan, M. T.** 1988. Microbiology, physiology and ecology of phototrophic bacteria, p. 39–111. *In* A. J. B. Zehnder (ed.), Biology of anaerobic micro-

organisms. John Wiley & Sons, Inc., New York.

17. **Méndez-Alvarez, S., V. Pavón, I. Esteve, R. Guerrero, and N. Gaju.** 1994. Transformation of *Chlorobium limicola* by a plasmid that confers the ability to utilize thiosulfate. J. Bacteriol. **176:**7395–7397.
18. **Miller, M., R. P. Cox, and J. M. Olson.** 1994. Low temperature spectroscopy of isolated FMO-protein and a membrane-free reaction center complex from the green sulfur bacterium *Chlorobium tepidum*. Photosynth. Res. **41:**97–103.
19. **Nozawa, T., K. Ohtomo, M. Suzuki, H. Nakagawa, Y. Shikama, H. Konami, and Z.-Y. Wang.** 1994. Structures of chlorosomes and aggregated bacteriochlorophyll *c* in *Chlorobium tepidum* from solid state high resolution CP/MAS ^{13}C NMR. Photosynth. Res. **41:**211–223.
20. **Okumura, N., K. Shimada, and K. Matsuura.** 1994. Photo-oxidation of membrane-bound and soluble cytochrome *c* in the green sulfur bacterium, *Chlorobium tepidum*. Photosynth. Res. **41:**125–134.
21. **Ormerod, J. G.** 1988. Natural genetic transformation in *Chlorobium*, p. 315–319. *In* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (ed.), Green photosynthetic bacteria. Plenum Press, New York.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. I–III. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Schütz, M., S. Zirngibl, J. le Coutre, M. Büttner, D.-L. Xie, N. Nelson, R. Deutzmann, and G. Hauska.** 1994. A transcription unit for the Rieske FeS-protein and cytochrome *b* in *Chlorobium limicola*. Photosynth. Res. **39:**163–174.
24. **Sharpe, G. S.** 1984. Broad host range cloning vectors for Gram-negative bacteria. Gene **29:**93–102.
25. **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:**784–791.
26. **Wahlund, T. M., and M. T. Madigan.** 1993. Nitrogen fixation by the thermophilic green sulfur bacterium *Chlorobium tepidum*. J. Bacteriol. **175:**474–478.
27. **Wahlund, T. M., C. R. Woese, R. W. Castenholz, and M. T. Madigan.** 1991. A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. Arch. Microbiol. **156:**81–90.
28. **Willison, J. C.** 1993. Biochemical genetics revisited: the use of mutants to study carbon and nitrogen metabolism in the photosynthetic bacteria. FEMS Microbiol. Rev. **104:**1–38.
29. **Wolk, C. P., A. Vonshak, P. Kehoe, and J. Elhai.** 1984. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. Proc. Natl. Acad. Sci. USA **81:**1561–1565.
30. **Xie, D.-L., H. Lill, G. Hauska, M. Maeda, M. Futai, and N. Nelson.** 1993. The *apt2* operon of the green bacterium *Chlorobium limicola*. Biochim. Biophys. Acta **1172:**267–273.
31. **Zhou, W., R. LoBrutto, S. Lin, and R. E. Blankenship.** 1993. Redox effects on the bacteriochlorophyll *a*-containing Fenna-Matthews-Olson protein from *Chlorobium tepidum*. Photosynth. Res. **41:**89–96.