Directed Mutagenesis and Plasmid-Based Complementation in the Methanogenic Archaeon *Methanosarcina acetivorans* C2A Demonstrated by Genetic Analysis of Proline Biosynthesis

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We report here the first use of directed mutagenesis in *Methanosarcina acetivorans* C2A. The method employs homologous recombination-mediated gene replacement and was used to construct a variety of proline auxotrophs with mutations in the *proABC* locus. Each mutation was also complemented in *trans* with autonomously replicating *Methanosarcina-Escherichia* plasmid shuttle vectors.

Studies of methane-producing archaea have been hampered by a lack of genetic tools; however, in recent years this has been changing rapidly. Useful genetic tools have now been developed for organisms in two genera, *Methanococcus* and *Methanosarcina*. These tools include selectable markers, transformation methods, plasmids, reporter genes, and a variety of mutagenesis protocols (reviewed in references 13 and 23). Despite these advances, serious deficiencies in the genetic tools available for many methanoarchaea are still evident. In particular, robust protocols for directed mutagenesis of *Methanosarcina* species, which are the most metabolically diverse of the methanoarchaea, are still lacking, while plasmid-based complementation of mutations has yet to be reported for any methanogen.

Directed mutagenesis of *Methanosarcina* species is clearly possible. Transformation and integration of foreign DNA into the chromosome via homologous recombination have been demonstrated for *Methanosarcina mazei* S-6 (6). In that study, the first to demonstrate the transformation of any *Methanosarcina* species, a selectable marker inserted into the *grpE-dnaK* intergenic region was recombined onto the *M. mazei* chromosome after chemical transformation. The process was, however, inefficient, requiring 50 μg of DNA to produce transformed cultures. More recently, a very efficient liposome-mediated transformation method for *Methanosarcina* species was developed. This method allows isolation of up to 10^8 transformants per μg of DNA in *Methanosarcina acetivorans* C2A. The availability of this high-frequency transformation method led us to attempt directed mutagenesis in this species. To examine the factors involved in this type of experiment and to verify that the process of homologous recombination occurs in a reliable and predictable manner, we decided to mutagenize genes with known functions and predictable phenotypes. For this purpose, we chose to examine genes involved in the biosynthesis of proline.

Cloning the proline biosynthetic genes of *M. acetivorans*. Previous studies demonstrated the feasibility of cloning proline biosynthetic genes from methanoarchaea by complementation of *Escherichia coli* mutants (10). Therefore, we used the same method to clone the *proC* gene from each of the three *Methanosarcina* species frequently used in our laboratory. Chromosomal DNAs, isolated as previously described (3) from *M. acetivorans* C2A, *Methanosarcina thermophila* TM1, and *Methanosarcina barkeri* Fusaro, were used to construct genomic libraries in the single-copy, dual-*cos* vector pWM357 (Table 1). The libraries contain ca. 330,000, 14,000, and 50,000 independent clones, respectively, and were prepared essentially as described previously (2). Numerous cosmids carrying the *pro* locus from each organism were subsequently obtained as plasmids that allow growth of the *E. coli proC* mutant XS72 on glucose-MOPS (morpholinepropanesulfonic acid) agar (19) without added proline (Pro$^+$ phenotype).

Two cosmids from each organism were moved into an isogenic set of *Salmonella enterica* serovar Typhimurium *proA*, *proB*, and *proC* mutants. Surprisingly, all of the cosmids complemented not only the *proC* mutation but also the *proA* and *proB* mutations, suggesting that each carries functional homologs of all three proline biosynthetic genes or a gene(s) that allows synthesis of proline via an alternate route. To address this issue, we carried out a series of subcloning and complementation experiments using one of the cosmids (pJK201) isolated from *M. acetivorans*. Random subclones of pJK201 were generated as described in Table 1. Pro$^+$ subclones were then isolated based on their ability to complement the *proA* mutant *Salmonella* serovar Typhimurium MST951(pAW3 and pAW4), the *proB* mutant *Salmonella* serovar Typhimurium MST952(pAW1 and pAW2), or the *proC* mutant *E. coli* BW10244(pCK1 and pCK2). In all cases, the subclones isolated by complementation of a particular *pro* mutation failed to complement the other two *pro* mutations, strongly supporting the conclusion that proline biosynthesis in *M. acetivorans* occurs via the same three-step pathway used by *Salmonella* (5). The fact that similar clones were isolated from *M. barkeri* and *M. thermophila* suggests that the same is true in these species as well.

DNA sequence analysis of the *M. acetivorans proABC* locus. The DNA sequences of the inserts in each of the pJK201 subclones were determined by automated dye terminator sequencing at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois. Upon analysis, these sequences could be compiled into a single continuous

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TABLE 1. Microorganisms and plasmids used in the study

Species and strain or plasmid	Relevant characteristic(s) or description and/or construction[b]	Source or reference
Escherichia coli		
DH5α	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	8
DH5α/λ*pir*	λ*pir*/φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	18
DH10B	φ80d*lacZ*αM15/*araD139* Δ(*ara leu*)7697 Δ*lacX74 galU galK rpsL deoR endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*)	8
BW10244	*proC*::Tn5-132	15
BW21116	Δ(*lacZYA-argF*)U169 *creC510 hsdR514 uidA*(Δ*Mlu*I)::*pir⁺*	14
XS72[a]	Δ(*lacZYA-argF*)U169 *creC510 hsdR514 uidA*(Δ*Mlu*I)::*pir⁺ proC*::Tn5-132	This study
GM119	*dam3 dcm9 metB1 galK2 galT27 lacY1 tsx-78 supE44 thi-1 mel1 tonA31*	21
Salmonella enterica serovar Typhimurium		
MST951	*proA41*	11
MST952	*proB8*	11
MST953	*proC90*	11
TR5877	*hsdL6 hsdSA29 ilv-452 metA22 trpC2 metE551 xly-404 fla-66 rpsL120* H1-b H2-e,n,x (Fels2)⁻*nml*	20
M. acetivorans C2A	Wild type (DSM2834)	Lab stock
M. thermophila TM1	Wild type (DSM1825)	Lab stock
M. barkeri Fusaro	Wild type (DSM804)	Lab stock
Plasmid		
pBace3.6	Cm^r, *oriF* vector	7
Supercos1	Dual *cos* vector	Stratagene; La Joya, Calif.
pBluescript KS(+)	Ap^r cloning vector	Stratagene; La Joya, Calif.
pSL1180	Ap^r cloning vector	4
pPB35	Pa^r Ap^r *Methanosarcina-Escherichia* shuttle vector	3
pWM321	Pu^r Ap^r *Methanosarcina-Escherichia* shuttle vector	16
pJK5	*pac-ori-aph* cassette plasmid	24
pBEND2	Ap^r symmetrical polylinker vector	12
pPB12	Pa^r Ap^r *Methanosarcina/Escherichia* shuttle vector	3
pWM353	*Eco*47III deletion of Supercos1	This study
pWM357	*oriF*, dual *cos* vector; *Xho*I-cut (added restriction sites shown in bold) dual-*cos* PCR fragment from pWM353 using primers CGCGCG**CTCGAG**CCTATAAAAATAGGCGTATCACGAGG and CGCGCG**CTCGAG**TTGAAGGCTCTCAAGGGCATCGGTCG ligated to a 6.3-kbp *Sal*I fragment from pBace3.6	This study
pJK201	Pro⁺ clone from *M. acetivorans* in pWM367	This study
pAW1	*proB*-complementing subclone; *Sau*3AI partial of pJK201 in *Bam*HI-cut pBluescript KS(+)	This study
pAW2	*proB*-complementing subclone; *Sau*3AI partial of pJK201 in *Bam*HI-cut pBluescript KS(+)	This study
pAW3	*proA*-complementing subclone; *Sau*3AI partial of pJK201 in *Bam*HI-cut pBluescript KS(+)	This study
pAW4	*proA*-complementing subclone; *Sau*3AI partial of pJK201 in *Bam*HI-cut pBluescript KS(+)	This study
pCK1	*proC*-complementing subclone; *Sau*3AI partial of pJK201 *Bam*HI cut in pSL1180	This study
pCK2	*proC*-complementing subclone; *Sau*3AI partial of pJK201 *Bam*HI cut in pSL1180	This study
pJK68	*M. acetivorans proABC* subclone; 4,611-bp *Nde*I of pJK201 into *Nde*I-cut pSL1180	This study
pJK69	*M. acetivorans proABC* subclone; 4,611-bp *Nde*I of pJK201 into *Nde*I-cut pSL1180 (orientation opposite to that of pJK68)	This study
pJK72	*M. acetivorans proABC* subclone; *Not*I-to-*Avr*II insert from pJK68 into *Not*I- and *Avr*II-cut pWM321	This study
pPB36	*M. acetivorans proABC* subclone; *Bam*HI-to-*Mlu*I insert from pJK72 into *Bam*HI- and *Mlu*I-cut pPB35	This study
pJK74	*M. acetivorans proAB* subclone; *Kpn*I deletion of pJK72	This study
pJK75	*M. acetivorans proA* subclone; *Bcl*I deletion of pJK72	This study
pJK76	*M. acetivorans proA* subclone; *Bcl*I deletion of pPB36	This study
pWM403	Ap^r vector with unique *Pvu*II site; *Pvu*II deletion of pBluescript KS(+)	This study
pWM404	*Pvu*II-cut PCR product of EZ::TN <KM-1> (Epicentre, Madison, Wis.) with the primer GAATT**CCAGCTG**TCTCTTATACACATCTCAACC into *Pvu*II-cut pWM403	This study
pWM407	2.9-kbp *Kpn*I-to-*Xba*I *pac-ori-aph* cassette of pJK5 into *Kpn*I-*Xba*I-deleted pWM404	This study
pTn5-407	Tn5-407 vector; circularization of the *Pvu*II fragment from pWM407	This study
pJK70	Tn5-*ileS12* vector; the *Xba*I-*Sph*I fragment from pPB12 was cloned into *Xba*I-*Sph*I-digested pWM404	This study
pJK71	Tn5-*ileS12* vector; the *Xba*I-*Sph*I fragment from pPB12 was cloned into the *Xba*I-*Sph*I-digested pWM405	This study
pJK61	*pac-ori-aph* cassette plasmid with symmetrical flanking sites; *Xba*I fragment of pJK5 into *Xba*I-cut pBend2	This study
pCK11	*Bam*HI fragment of pCK1 into *Bam*HI-cut pBluescript KS(+)	This study
pCK12	*Bam*HI fragment of pCK1 into *Bam*HI-cut pBluescript KS(+) (orientation opposite to that of pCK11)	This study
pCK14	*proC1*::*pac-ori-aph* plasmid; *Bam*HI cassette from pJK61 into *Bgl*II-cut pCK12	This study
pCK16	*proC2*::*pac-ori-aph* plasmid; *Bam*HI cassette from pJK61 into *Bgl*II-cut pCK11	This study

Continued on following page

Species and strain or plasmid	Relevant characteristic(s) or description and/or construction[b]	Source or reference
pJK68- *proA1*::Tn5-407	In vitro transposon mutagenesis of pJK68 with Tn5-407[c]	This study
pJK68- *proA2*::Tn5-407	In vitro transposon mutagenesis of pJK68 with Tn5-407[c]	This study
pJK68- *proA3*::Tn5-ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study
pJK68- *proA4*::Tn5-ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study
pJK68- *proB1*::Tn5-407	In vitro transposon mutagenesis of pJK68 with Tn5-407[c]	This study
pJK68- *proB2*::Tn5-ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study
pJK68- *proB3*::Tn5-ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study
pJK68- *proC3*::ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study
pJK68- *proC4*::ileS12	In vitro transposon mutagenesis of pJK68 with Tn5-ileS12[c]	This study

[a] The *proC*::Tn5-132 allele of BW10244 was moved into BW21116 by P1 *kc* transduction to construct XS72. Tetracycline-resistant transductants were selected on TYE-tetracycline agar (22) and screened for Pro⁻ phenotype on glucose-MOPS agar with and without proline.

[b] Standard methods were used throughout for isolation and manipulation of plasmid DNA from *E. coli* (1). *E. coli* strains DH5α and DH10B were used as the hosts for most cloning experiments; DH5α/λ*pir* was used as the host for cloning experiments involving *pir*-dependent replicons. Plasmids used for constructions involving *Bcl*I were isolated from *E. coli* GM119. Plasmids to be transformed into *Salmonella* serovar Typhimurium that were originally isolated from *E. coli* were usually passed through *Salmonella* serovar Typhimurium TR5877 first, to avoid problems associated with host restriction systems.

[c] In vitro mutagenesis was performed using EZ::TN transposase (Epicentre) as recommended. Transposon Tn5-407 as used in these reactions was prepared by linearization of pTn5-407 with *Pvu*II; transposon Tn5-ileS12 was prepared by purification of the 6.8-kbp *Fsp*I fragment from either pJK70 or pJK71. Mutagenized plasmids were recovered from Amp^r and Kan^r transformants of DH10B. The orientation and insertion site of each mutation were verified by DNA sequencing and are shown in Fig. 1. The primers used for determining the DNA sequences of transposon insertion junctions were pJK5forI (5′-GTATATTACGAATAGGGCG-3′) and pJK5revI (5′-AGCTGCTGGTGAAAGAGAC-3′) for Tn5-407 and EZTNkanrev (5′-GGTACCGAGCTCGAATTCAT-3′) and ileS12rev (5′-CGAGCAGGTGATGAAAAGG-3′) for Tn5-ileS12.

DNA fragment of 5,139 bp containing three large open reading frames, which, based on sequence analysis, were designated *proA*, *proB*, and *proC* (Fig. 1). The predicted protein products of the *proA*, *proB*, and *proC* genes show very significant homology to known ProA (glutamate semialdehyde dehydrogenase), ProB (glutamate kinase), and ProC (1-pyrroline-5-carboxylate reductase) proteins, with between 30 and 50% identities to the top 20 BLAST matches for all three proteins (see below). The results of these homology searches are in complete agreement with the complementation data presented above. Accordingly, the only complete gene found on the *proA*-complementing plasmids is *proA*, the only complete gene found on the *proB*-complementing plasmids is *proB*, and the only complete gene on the *proC*-complementing plasmids is
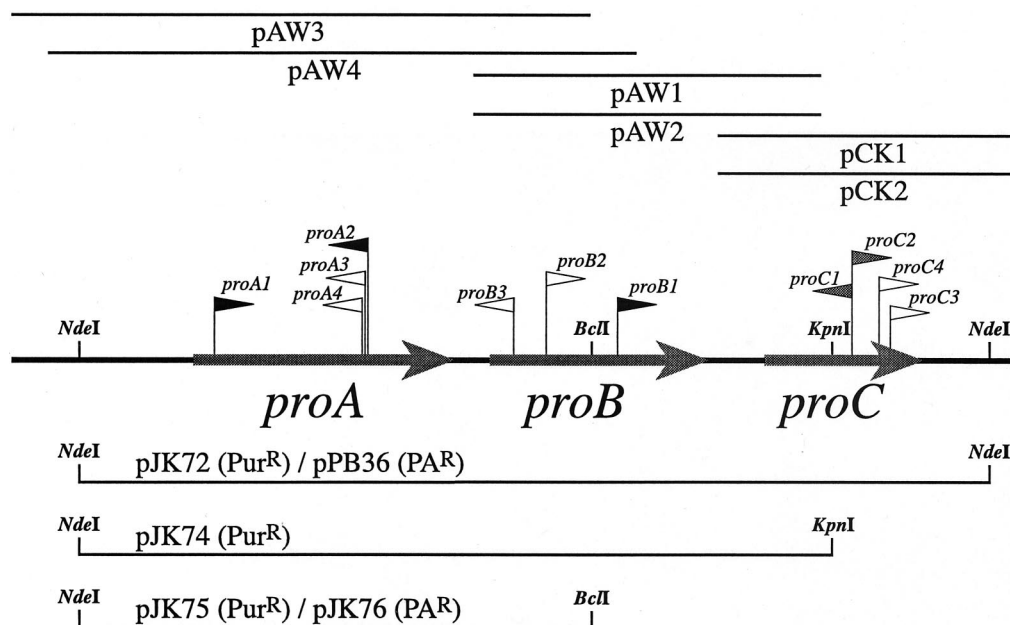

FIG. 1. The *M. acetivorans* C2A *proABC* locus. The physical structure of a 5,139-bp fragment of the *M. acetivorans* chromosome is shown in the center (heavy black line), with the positions of the *proA*, *proB*, and *proC* genes shown as gray arrows. The regions covered by the six plasmids whose DNA sequences were determined are shown as thin black lines at the top, with the names of the respective plasmids shown below each line. The position and relative orientation of each in vitro-constructed insertion mutation are indicated by a flag on the center line. The black flags represent Tn5-407 insertions, the white flags represent Tn5-ileS12 insertions, and the gray flags represent insertions of the *pac-ori-aph* cassette. The regions contained in several plasmid shuttle vectors used for complementation studies are shown as thin black lines at the bottom. The names of the respective plasmids and antibiotic resistance markers are shown above each line. The endonuclease restriction sites used for construction of these shuttle vectors are indicated.

proC (Fig. 1). Thus, in combination, these data provide strong support for the function of the genes in the *M. acetivorans* *proABC* locus, i.e., the biosynthesis of proline via the standard pathway found in most organisms.

Although the findings regarding the function of *M. acetivorans* genes in the standard proline biosynthesis pathway are not surprising per se, they are at odds with the situation observed for other archaea. Recent data suggest that proline is synthesized from ornithine in several methanoarchaea (9). Moreover, examination of the 10 complete archaeal genome sequences presently available failed to identify complete sets of genes required for the standard biochemical pathway (searches were performed in September 2001). Thus, recognizable ProA homologs were not observed (based on a BLAST E value of <0.04 when *M. acetivorans* ProA is used as the query sequence) in any of the completed archaeal genome sequences, which include those of *Aeropyrum pernix* K1, *Archaeoglobus fulgidus*, *Halobacterium* sp. strain NRC-1, *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Sulfolobus acidocaldarius*, *Thermoplasma acidophilum*, and *Thermoplasma volcanium*. In contrast, sequence comparisons of *M. acetivorans* ProA with dozens of other ProA proteins produced highly significant scores (the top 50 matches produced E values of $<4 \times 10^{-44}$); these proteins included many with biochemically and genetically demonstrated function. Using *M. acetivorans* ProB as the query sequence, a possible ProB homolog was identified in *Methanobacterium thermoautotrophicum* (E value of 3×10^{-4}) but not in the remaining archaeal genomes (based on E values of <0.01). When compared with the top 50 known and putative ProB proteins, *M. acetivorans* ProB produced scores of $<7 \times 10^{-20}$. In contrast, easily recognizable ProC proteins were found in 5 of the 10 available archaeal genomes. Thus, it appears that recognizable ProC homologs are more common, but not universal, among archaea. As described above, a *proC* gene was previously identified in *Methanobrevibacterium smithii* by genetic complementation (10), but this gene is not among the closest homologs to *M. acetivorans* *proC*. In sum, it is clear that the evolution of proline biosynthesis in *Methanosarcina* species is dramatically different than in the other members of the domain *Archaea* for which data are available; they are the only known archaea to possess the standard pathway for proline biosynthesis.

Directed mutagenesis of *M. acetivorans* proabc by homologous recombination-mediated allele replacement. A variety of *pro* mutations were constructed on plasmids for subsequent recombination onto the *M. acetivorans* chromosome as described in Table 1. These gene disruption mutations consist of those with insertions of either the *pac* gene (in the form of the *pac-ori-aph* cassette or Tn5-407) or the *ileS12* gene (in the form of Tn5-ileS12), which encode resistance to puromycin or pseudomonic acid, respectively, in *Methanosarcina* species (3, 16). Both transposons and the *pac-ori-aph* cassette also encode resistance to kanamycin in *E. coli* to simplify the plasmid constructions. The sites and orientations of each insertion mutation are shown in Fig. 1.

Each of the plasmid-borne *pro* alleles was transferred onto the *M. acetivorans* chromosome by simple homologous recombination after liposome-mediated transformation (3, 16) with linear DNA. For these experiments, the plasmids were linearized with restriction enzymes that cut only the plasmid backbone, as close to the insert as possible. Recombinants carrying Tn5-407 or *pac-ori-aph* cassette insertions were selected as puromycin-resistant (Pur[r]) clones; recombinants carrying Tn5-ileS12 insertions were selected as pseudomonic acid-resistant (PA[r]) clones. In both cases, selection for recombinants was carried out in medium containing 50 mM proline to allow growth of the expected auxotrophic mutants. (There is no available evidence to suggest that proline is transported by *M. acetivorans*. We used this relatively high proline concentration in the hope of overcoming potential transport defects.) In these experiments, transformation with ca. 2 μg of linearized plasmid DNA yielded from a few hundred to a few thousand antibiotic-resistant colonies. After purification of the recombinant clones by streaking for single colonies on solid-medium plates, 80 to 100% of these transformants (20 were tested for each mutation) required proline for growth (Pro[−] phenotype). All 11 of the *pro* mutations yielded Pro[−] transformants.

Verification of the chromosomal structure of the *M. acetivorans* Pro[−] mutants. Because allele replacement has not previously been employed in *M. acetivorans*, we examined the physical structure of each mutant chromosome in detail by using DNA hybridization. DNA hybridizations were performed essentially as described previously (17); however, a digoxigenin-labeled probe (pJK68) and chemiluminescence detection (DIG High Prime and CDP-Star, respectively; Roche Molecular Biochemicals, Indianapolis, Ind.) were used for visualization of hybridizing bands in place of ^{31}P-labeled probes. For all 11 *M. acetivorans* Pro[−] mutants, there was complete agreement between the sizes of the hybridizing bands predicted from the known DNA sequences and those obtained experimentally (data not shown). Thus, the mutant strains that we constructed were indistinguishable from those that would result from simple homologous recombination, and this finding provides strong support for the occurrence of this genetic process in *M. acetivorans* C2A.

Plasmid-based complementation of *M. acetivorans* proABC mutants. A series of *Methanosarcina-E. coli* shuttle vectors have been reported (3, 16); however, these have never been used for complementation studies because *Methanosarcina* mutants were not previously available. To examine whether plasmid-borne alleles could complement genomic mutations, each of the PA[r] Pro[−] mutants was transformed with a puromycin resistance plasmid, pJK72, carrying the entire *proABC* locus. The shuttle vector pWM321 (with no insert) was used as a negative control. In a similar way, each of the Pur[r] Pro[−] mutants was transformed with the pseudomonic acid resistance *proABC* plasmid, pPB36, by using the shuttle vector pPB35 as a control (Table 2). All of the strains transformed by the *proABC* plasmids regained the Pro[+] phenotype, indicating that each chromosomal mutation was complemented by the plasmid-borne, wild-type allele. Strains transformed by the vector controls remained Pro[−]. Because these mutant strains are recombination proficient, it is possible that restoration of the Pro[+] phenotype occurred by marker rescue (i.e., recombinational repair) rather than by true complementation. However, we do not believe this was the case. Selection for both antibiotics was maintained throughout the experiment. Therefore, the antibiotic resistance marker on the chromosome was not removed by recombination unless a reciprocal recombination

TABLE 2. Plasmid complementation of *M. acetivorans*
proABC mutations[a]

Mutation	Complementation with:			
	Vector only	*proABC*	*proAB*	*proA*
	pPB35	pPB36	None	pJK76
proA1::Tn5-407	−	+	ND[b]	+
proA2::Tn5-407	−	+	ND	+
proB1::Tn5-407	−	+	ND	ND
proC2::pac-ori-aph	−	+	ND	ND
proC1::aph-ori-pac	−	+	ND	ND
	pWM321	pJK72	pJK74	pJK75
proA3::Tn5-*ileS12*	−	+	+	+
proA4::Tn5-*ileS12*	−	+	+	+
proB2::Tn5-*ileS12*	−	+	+	ND
proB3::Tn5-*ileS12*	−	+	+	ND
proC3::Tn5-*ileS12*	−	+	ND	ND
proC4::Tn5-*ileS12*	−	+	ND	ND

[a] Complementation was scored according to ability to grow on HA-MA medium with puromycin and pseudomonic acid but without proline. Plasmids used are described in the text and shown in Fig. 1.
[b] ND, not done.

event exchanged the markers on the plasmid and the chromosome. In each case, we were able to recover the intact original plasmid from the complemented strain, indicating that no such event had occurred (data not shown).

Although all the mutations tested resulted in proline auxotrophy, it is not clear from mutant analysis alone whether *proA* and *proB* have a role in proline biosynthesis. Given the potential operon structure of the *proABC* locus, it was formally possible (although perhaps unlikely) that only *proC* was required for proline biosynthesis and that the effects of the *proA* and *proB* mutations were via polarity on *proC*. Accordingly, we transformed the *proA*::Tn5-*ileS12* mutants with puromycin resistance plasmids carrying only *proA* (to check for polarity on both *proB* and *proC*) or *proAB* (to check for polarity on *proC*). In a similar way, the *proB*::Tn5-*ileS12* mutants were transformed with a puromycin resistance plasmid carrying only *proAB* to check for polarity on *proC* (Table 2). In all cases, the plasmids were able to complement the transposon insertion mutations, indicating that these Tn5-*ileS12* mutations were not polar on the downstream genes and that *proA* and *proB* are required for proline biosynthesis in *M. acetivorans*. Similar experiments involving complementation of Tn5-407 insertions by pseudomonic resistance plasmids yielded identical results (Table 2).

As discussed above, the ability to complement chromosomal mutations by plasmid-borne alleles was critical in defining the functions of *proA* and *proB*. To our knowledge, this is the first example of plasmid complementation in a methanogenic archaeon. The ability to use plasmids in this way will greatly expand our ability to perform genetic analysis in *Methanosarcina* species. Most importantly, plasmid complementation will allow verification that a particular phenotype is caused by a mutation in a specific gene (namely, the one carried on the complementing plasmid). However, innumerable genetic techniques require the use of replicating plasmids. Among these are the identification of essential genes and regulatory proteins and the introduction of foreign genes, or genes carrying defined mutations, into strain backgrounds with mutated alleles on the chromosome.

The use of plasmids for complementation of mutations created by the insertion of selectable markers requires, of course, multiple selectable markers. Currently, only two such markers, the *ileS12* gene for resistance to pseudomonic acid (3) and the *pac* cassette for resistance to puromycin (16), are available for use in *Methanosarcina*. Our demonstration that plasmids carrying proline genes can complement proline auxotrophic mutations suggests the possibility of developing a third selectable marker from these genes. This would require a suitable proline auxotroph as a host strain, preferably one that did not already carry an antibiotic insertion in the chromosome. Experiments designed to develop such a system are presently under way in our laboratory.

These new techniques for directed mutagenesis and plasmid complementation, along with the recently presented method for random transposon-based mutagenesis (24), now complete a set of basic tools for genetic analysis of *Methanosarcina* species. These tools, in combination with the metabolic diversity of members of this genus (relative to other methanoarchaea), make *Methanosarcina* species very attractive model organisms for the study of methanogenesis and of archaeal biology in general.

Nucleotide sequence accession number. The sequence for the *M. acetivorans* C2A *proABC* locus has been deposited in GenBank under accession number AF305580.

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