CLONING OF A GENE FROM *STREPTOMYCES* SPECIES COMPLEMENTING *arg*G MUTATIONS

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(Received for publication December 6, 1984)

A gene that complements *arg*G mutations was cloned from *Streptomyces coelicolor* and *Streptomyces lividans* by using pIJ702 as a vector. The recombinant plasmid pMCP25 complemented *arg*G mutations of *S. lividans* and *S. coelicolor*. The inserted DNA of pMCP25 was able to hybridize with *arg*G$^+$ strains of *S. lividans*, *S. coelicolor*, *S. lavendulae* and *S. alboniger* but not with *arg*G mutants of these strains.

Most species of *Streptomyces* frequently give ArgG$^-$ mutants which require arginine or argininosuccinate. This type of mutant shows peculiar characteristics compared with the other auxotrophic mutants. Firstly, there are interesting relationships between the phenotype of the *arg*G mutation and other phenotypes in *Streptomyces*, such as aerial mycelium formation[1,2] or chloramphenicol sensitivity[3,4]. These indicate that there is some relationships between the *arg*G mutation and secondary metabolism in *Streptomyces*. Secondarily, in most of the cases the *arg*G mutation was nonrevertible. This suggests that the *arg*G mutation in *Streptomyces* is due to the deletion of a DNA fragment. ALTENBUCHNER and CULLUM reported that spontaneous chloramphenicol sensitive mutants of *Streptomyces lividans* produced Amy$^-$ ArgG$^-$ mutants at high frequency and that the ArgG$^-$ mutants contained amplified DNA sequences[3]. Both deletions and amplifications are known to cause mutation of other unstable genes in *Streptomyces*[5,6].

Recently, the *arg*G gene was successfully cloned from *S. cattleya* in *Escherichia coli* by STREICHER and the gene was able to convert *arg*G Amy$^-$ clones to Amy$^+$ prototrophy (personal communication). By SOUTHERN blot hybridization with a plasmid which contained the *arg*G gene from *S. cattleya* cloned into pBR322, LIU *et al.* reported that a complete deletion of the *arg*G gene was found in *arg*G Amy$^-$ isolates of some *Streptomyces* (B. M. POGELL, personal communication).

Here we describe the cloning of *arg*G complementary DNA from *S. coelicolor* and *S. lividans* and show that the *arg*G mutation occurs by the deletion of the cloned gene in several *Streptomyces* strains.

Materials and Methods

Strains, Media, Plasmid and Phage

Plasmid pIJ702[7] was a gift from D. A. HOPWOOD. Strains used in this paper are listed in Table 1. Arg$^-$ derivatives of *S. lividans* 1326 were obtained by nitrosoguanidine treatment[8]. Phage vector R4L24 was described previously[9,10].

YMS medium (1% malt extract, 0.4% yeast extract, 0.4% soluble starch, 2% agar, pH 7.5 adjusted with KOH) was used as a complete nutritional medium. MM[8] was used as a minimal nutritional medium. R2P medium excluding Casamino Acids from R2 medium[11] was used as a minimal nutritional protoplast regeneration medium. If needed, amino acids were supplemented to a final concentration of 100 μg/ml.

Table 1. Lists of strains used in this paper.

Strains	Genetic markers	Source
Streptomyces coelicolor A3(2) M130	*his*A1, *ura*A1, *str*A1	*1
S. coelicolor A3(2) 39	*his*A1	*2
S. coelicolor A3(2) 509b	*arg*G, *arg*A1, *ura*A1, *cmls*	*2
S. coelicolor A3(2) 512	*arg*G, *met*A2, *phe*A1, *cmls*	*2
S. coelicolor A3(2) 558	*arg*G *his*D3, *phe*A1, *cmls*	*2
S. coelicolor S0691	wild	*3
S. coelicolor S0979	wild	*3
S. lividans 1326	wild	*1
S. lividans 1326 I01	*arg*	*4
S. lividans 1326 I10	*arg*G	*4
S. alboniger S0309	wild (derived from ATCC 12461)	*3
S. alboniger 12461/5	*arg*G, *amy*	*5
S. lavendulae S0985	wild	*3
S. lavendulae S0055-B1	wild	*3
S. lavendulae S0055-B1 11-3	*arg*G, *amy*	*6

*1 Gift from D. A. HOPWOOD. *2 Gift from G. SERMONTI and M. R. MICHELI. *3 Gift from A. SEINO. *4 This paper. *5 Gift from B. M. POGELL. *6 Discussed in ref 10.

DNA Preparation

The isolation of the total cellular DNA was carried out using a modification of the method of MARMUR[12]. Plasmid DNA was prepared by the rapid alkaline denaturation procedure described by IKEDA *et al.*[13], and purified by CsCl-ethidium bromide (EtBr) density gradient centrifugation or by electroelution from agarose gels.

Cloning of *arg*G Complementary DNA

S. coelicolor M130 total DNA was digested partially with *Bgl* II and mixed with a *Bgl* II digest of pIJ702 plasmid DNA. The mixture then was ligated with T4 DNA-ligase and transformed into *S. lividans* 1326 I10 protoplasts. Only *arg*G$^+$ clones were able to grow on the R2P regeneration medium.

SOUTHERN Hybridization

As a probe the inserted DNA fragment of pMCP25 was used, which was isolated by electroelution from 4% polyacrylamide gels after digestion with *Bgl* II. The DNA was labeled by the nick translation method using [α-^{32}P]dCTP (approximately 3,000 Ci/mmol; Amersham, Japan). Chromosomal and extrachromosomal DNAs were digested with restriction enzymes and electrophoresed on 0.7% or 1.5% agarose gels. DNAs separated on the agarose gels were transferred to nitrocellulose paper by the method of SOUTHERN[14]. The nitrocellulose filter blots were incubated at 52°C for at least 2 hours in prehybridization buffer (6×SSC-1×Denhardt solution - 20 μg/ml tRNA - 50% formamide) and then incubated at 52°C for 40 hours in hybridization buffer (6×SSC-1×Denhardt solution - 0.5% SDS - 10 mM sodium pyrophosphate - 50% formamide) with the probe DNA. After hybridization, the blots were rinsed with 6×SSC-1×Denhardt solution - 50% formamide and incubated at 52°C for 1 hour in the same solution. The blots were then rinsed and incubated at 52°C for 30 minutes with 6×SSC, followed by washing and incubating with 2×SSC. The last step was repeated three times. The dried blots were exposed to X-ray film (Type RX Fuji Film, Japan) with an intensifying screen at −70°C.

Results

I10 Has *arg*G Phenotype

Several auxotrophic mutants were isolated from *S. lividans* 1326 after mutagenesis with nitrosoguanidine. The ArgG$^-$ mutants, which were obtained frequently, were classified into two groups by their nutritional requirements. The first group, typically I10, required either arginine or argininosuc-

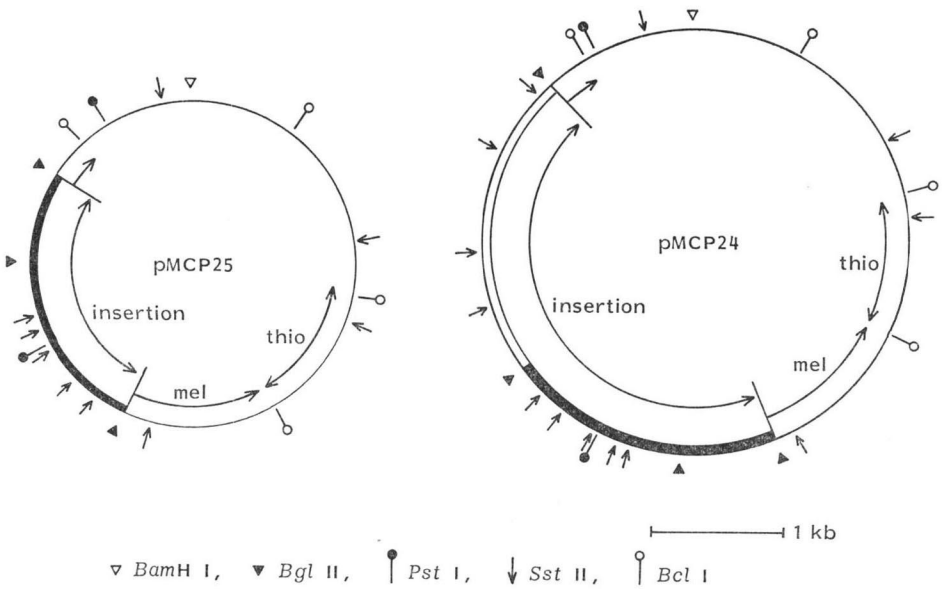
Table 2. Cloning of *arg*G gene from *S. coelicolor* and *S. lividans*.

Expt	No.	Donor DNA*	Vector DNA**	Number of *arg*G$^+$ clones	pMCP24/pMCP25
A	1	M130 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	9	2/7
	2	M130 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	8	0/8
	3	M130 0.2 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	30	2/28
	4	None	pIJ702/*Bgl* II 0.8 μg	2	0/2
B	5	M130 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	8	0/4
	6	M130 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	7	0/4
	7	1326 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	12	0/4
	8	1326 1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	5	0/4
	9	1326 0.1 μg/*Bgl* II	pIJ702/*Bgl* II 0.2 μg	180	0/4
	10	None	pIJ702/*Bgl* II 0.2 μg	0	—
	11	None	pIJ702/*Bgl* II 0.8 μg	0	—

* Donor DNAs were isolated by the modified method of MARMUR except Expt 9 in which donor DNA was prepared by the alkaline denaturation method.

** Vector plasmid was isolated by the alkaline denaturation method from strain 1326 (Expt A) or strain I10 (Expt B).

Fig. 1. Restriction maps of pMCP25 and pMCP24.



cinate. The second group, typically I01, required either ornithine, citrulline, or arginine. This implied that the former strains such as I10 show the ArgG$^-$ phenotype.

We could not isolate any revertant from 12 ArgG$^-$ mutants like I10, although 12 Arg$^-$ mutants of the second group showed reversion at a frequency of 10^{-6} to 10^{-7}. The ArgG$^-$ mutants simultaneously were conditionally aerial mycelium negative (Amy*); they formed aerial mycelium only on media containing a high concentration of arginine (5~100 mM).

Cloning of *arg*G Complementary DNA on Recombinant Plasmids pMCP24 and pMCP25

Table 2A summarizes the results of cloning of the *arg*G complementary gene. Of 47 *arg*G$^+$ clones,

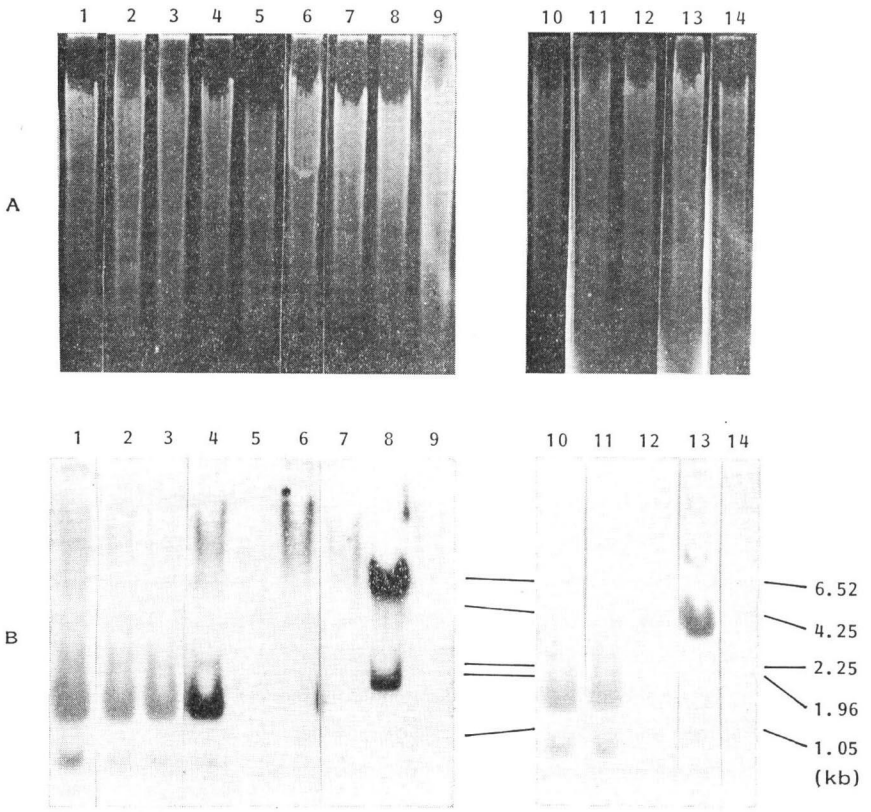
Table 3. Genotype of host strains before and after transformation with pMCP25.

Strains	Genotype	
	Before transformation	After transformation
Streptomyces lividans 1326		
I01	*arg*	*arg*
I10	*arg*G *amy**	*amy*$^+$
S. coelicolor A3(2)		
509b	*arg*G *arg*A1 *ura*A1 *cmls amy**	*arg*A1 *ura*A1 *cmls amy*$^+$
512	*arg*G *met*A2 *phe*A1 *cmls*	*met*A2 *phe*A1 *cmls*
558	*arg*G *his*D3 *phe*A1 *cmls*	*his*D3 *phe*A1 *cmls*

Fig. 2. Electrophoretic patterns of chromosomal DNA and corresponding autoradiograms.

Total DNAs were digested with *Bgl* II and run on 0.7 % agarose gels (A). The DNAs were transferred to nitrocellulose filters and hybridized with ^{32}P-labeled pMCP25 inserted DNA (B).

Lane 1~7: *S. coelicolor*; M130, S0691, S0979, 39, 509b, 512 and 558, respectively. Lane 8, 9: *S. alboniger*; S0309 and 12461/5, respectively. Lane 10~12: *S. lividans*; 1326, I01 and I10, respectively. Lane 13, 14: *S. lavendulae*; S0055-B1 and S0055-B1 11-3, respectively.



about 90% contained DNA identical to pMCP25 and the rest contained plasmid identical to pMCP24. Vector DNA only which was self-ligated after cleavage with *Bgl* II gave 2 *arg*G$^+$ transformants. This will be mentioned later. Restriction enzyme maps of pMCP24 and pMCP25 are shown in Fig. 1 from which it can be seen that the two plasmids have a common area in their DNA insertions. The whole insertional fragment of pMCP25 was identical to a part of the insertional fragment of pMCP24 (thick

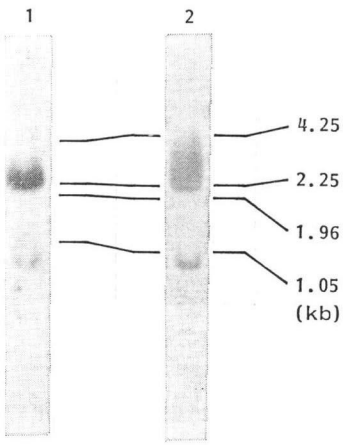
line of circle in Fig. 1) except that this insertion had an opposite orientation. As both plasmids complemented the ArgG$^-$ phenotype of strain I10, the extra DNA present in pMCP24 is not essential for *arg*G complementation.

Plasmid curing of 110 possessing pMCP25 was accomplished by protoplast regeneration. All of the cured clones had the ArgG$^-$ phenotype. The *arg*G$^+$ transformants were Amy$^+$ and the cured clones were Amy*.

Fig. 3. Analysis of *arg*G in total DNA of *S. lividans* and *S. coelicolor*.

Total DNAs were digested with *Sst*II and run on 1.5% agarose gels and SOUTHERN blotting and hybridization experiment were done as described in Fig. 2.

Lane 1: *S. coelicolor* 39. Lane 2: *S. lividans* 1326.



Expression of Insertional DNA of pMCP25 in Phage Vector R4L24

The inserted DNA fragment of pMCP25 was embedded in a single *Bam*H I site of phage vector R4L24[9,10]. The recombinant phage particle, R4L24RB5, was obtained by transfection of *S. lavendulae* S0985 protoplasts at high frequency[10]. The phage was infected into strain I10 on the MM medium. After incubation for a week, lysogens growing on the MM plates showed an ArgG$^+$ phenotype. The vector phage and recombinant phages carrying other DNA fragments were unable to convert I10 to *arg*G$^+$.

Transformation of *S. coelicolor* with pMCP25

Various Arg$^-$ mutants derived from *S. coelicolor* A3(2) and *S. lividans* 1326 were transformed by plasmid pMCP25. Genotypes of the host strains are shown in Table 3. Transformants were selected by their resistance to thiostrepton and were examined for the restoration of *arg* prototrophy. Both *S. coelicolor* and *S. lividans arg*G mutations were complemented but mutations of other types were not (Table 3).

*arg*G Mutation Is Due to Deletion

Fig. 2 shows hybridization patterns of ^{32}P-labeled pMCP25 inserted DNA to *Bgl* II-digested fragments of total DNAs from several *Streptomyces* species. The probe hybridized with several fragments from *arg*G$^+$ strains of *S. coelicolor*, *S. lividans*, *S. alboniger* and *S. lavendulae*. However, DNAs from ArgG$^-$ mutants of these strains did not hybridize with the probe. This shows that the *arg*G mutation is due to the deletion of some DNA from wild type genomes. Similar hybridization patterns were observed between *S. coelicolor* and *S. lividans*. *Sst* II digests of the total DNA from the two strains also showed the same hybridization patterns (Fig. 3). It implied that the *arg*G complementary regions of *S. coelicolor* and *S. lividans* were identical at least in their cleavage patterns with *Bgl* II or *Sst* II. DNA digests from *S. lavendulae* S0055B1 and *S. alboniger* S0309 showed different hybridization patterns.

Origin of pMCP25 Inserted DNA

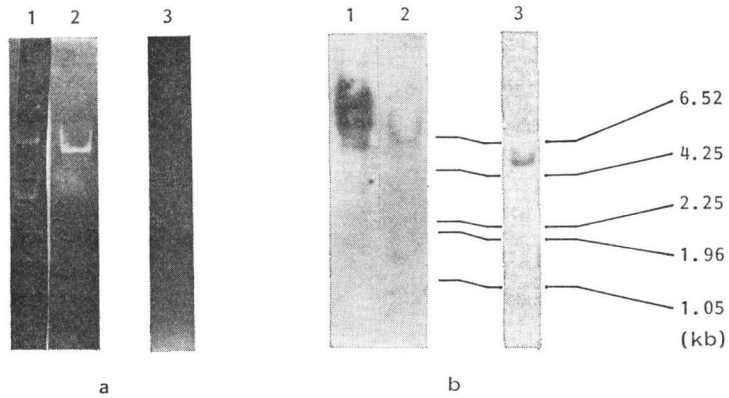
As the *arg*G genes of *S. coelicolor* M130 and *S. lividans* 1326 had the same cleavage patterns with two restriction enzymes, we tried to clone the *arg*G gene from *S. lividans* 1326 and compare it with that

Fig. 4. Existence of *arg*G complementary DNA in *S. lividans* 1326.

Plasmid pIJ702 was prepared from *S. lividans* 1326 possessing the plasmid by the alkaline denaturation method. DNA from strain 1326 was prepared by the same method as the plasmid preparation.

(a) 0.7 % agarose gel electrophoresis. Lane 1: pIJ702, not digested, Lane 2: pIJ702, digested with *Bgl* II, Lane 3: DNA from strain 1326 which did not contain pIJ702, not digested.

(b) Autoradiogram of SOUTHERN hybridization with the DNA insert from pMCP25.



of strain M130. Table 2B shows that 17 *arg*G$^+$ clones were isolated from total DNA prepared by the modified method of MARMUR from strain 1326. All of the clones were shown to have the pMCP25 plasmid.

As mentioned above, the *arg*G$^+$ clones were obtained by the transformation with only the vector DNA after self-ligation of its *Bgl* II digest and the recombinant plasmid pMCP25 was isolated from the *arg*G$^+$ transformants (Table 2A, Expt 4). The plasmid pIJ702 was isolated from strain 1326 carrying it by alkaline denaturation method followed by CsCl-EtBr buoyant centrifugation. However, in this purified plasmid DNA some DNA fragments appeared to hybridize with the inserted DNA of pMCP25 (Fig. 4a, b). When genomic DNA of strain 1326 without pIJ702 was isolated by the alkaline denaturation method, a band existed which hybridized with the DNA insertion of pMCP25 (Fig. 4b, lane 3), although no such band could be detected on electrophoresis (Fig. 4a, lane 3). These data suggest that a trace of some DNA which coexisted in the plasmid preparation might be digested with *Bgl* II and ligated with the vector DNA. This suggestion is supported by the fact that pIJ702 prepared from strain I10 did not give *arg*G$^+$ transformants at all (Table 2B, Expt 10 and 11). Table 2B shows that total DNA prepared from strain 1326 by the alkaline denaturation method (Expt 9) gave *arg*G$^+$ transformants at higher frequency than the same DNA prepared by the method of MARMUR (Expt 7 and 8). This might be caused by the tendency of the *arg*G gene to exist extrachromosomally; however, further studies are necessary to prove this.

Discussion

We have isolated *arg*G complementary DNA from *S. coelicolor* and *S. lividans*. The cloned *arg*G DNAs from both strains were indistinguishable by the cleavage patterns with restriction enzymes. These species show differences in stability of plasmid and plasmid profile[15]. However, there are few differences in their linkage maps[15] or in repeated DNA sequence profile[16]. Similarity of *arg*G complementary DNAs of these two species may relate to their phylogenetic closeness. *S. lavendulae* and *S. alboniger* are quite different phylogenetically from *S. coelicolor* and *S. lividans*. However, all of the DNAs from ArgG$^-$ mutants of these 4 strains were unable to hybridize with the pMCP25

inserted DNA, although DNAs from their ArgG$^+$ strains were able to hybridize. This suggests that deletion of the DNA we cloned may cause the *arg*G mutation in all four species. However, as compared with a similarity in hybridization patterns of *S. lividans* and *S. coelicolor*, those of *S. alboniger* and *S. lavendulae* were quite distinct. It is not clear at the present time whether this is derived from difference of the *arg*G gene's structure of these species or from an irrelevant event, because no transformants of pMCP25 nor stable lysogens of R4L24RB5 have been obtained yet in ArgG$^-$ mutants of *S. lavendulae* and *S. alboniger*.

The *arg*G mutation is unique in *Streptomyces*. In *S. lividans*, it is often accompanied by the Amy* phenotype and chloramphenicol sensitivity[3]. In *S. coelicolor*, the *arg*G mutation is associated with chloramphenicol sensitivity[4] and with Amy$^-$ phenotype[2]. In *S. lavendulae* and *S. alboniger*, the *arg*G mutation shows the Amy$^-$ phenotype[1,2]. The recombinant plasmid pMCP25 reported in this paper restored the Amy$^+$ phenotype and complemented the *arg*G mutation at least in *S. lividans* I10 and *S. coelicolor* 509b, while it did not restore chloramphenicol resistance (Table 3).

ALTENBUCHNER and CULLUM reported that ArgG$^-$ mutants were isolated at frequency of about 25% from chloramphenicol sensitive mutants of *S. lividans* 66 and that all the ArgG$^-$ mutants had an amplified DNA fragment[3]. We could not find such an amplified DNA in ArgG$^-$ mutants isolated in this paper. There is another interesting study which suggests the involvement of DNA deletion and amplification in genetic instability of *Streptomyces* strains. The ability to produce melanin in *Streptomyces* is also known to be frequently lost. SCHREMPF showed that melanin negative variants from *S. reticuli* were found to have lost tyrosinase gene and to contain amplified nucleotide sequences within their genomes, although it is unknown yet if amplification of specific nucleotide sequences is triggered by deletion events or *vice versa*[6]. SERMONTI *et al.* suggested possible involvement of a transposable element in chloramphenicol sensitive and Arg$^-$ mutants of *S. coelicolor* A3(2)[4]. This is an attractive hypothesis because transposable elements in eubacteria participate in chromosomal rearrangement such as gene deletion, translocation, inversion and duplication, and can control gene expression.

Although we can not conclude at present whether the cloned gene is the structural gene of argininosuccinate synthetase or a regulatory gene, the cloned gene will be useful tool to analyze not only the *arg*G mutation directly, but also other interesting behavior of *Streptomyces* connected with the ArgG$^-$ phenotype.

Acknowledgments

We thank Dr. MARIA R. MICHELI, Dr. GUISEPPE SERMONTI and Dr. AKIO SEINO for kind gifts of *Streptomyces* strains. We also thank Dr. DAVID A. HOPWOOD for kind gifts of *Streptomyces* strains and plasmids. Thanks are due to Dr. BRUTON M. POGELL for a kind gift of *Streptomyces* strains and for invaluable discussion, and Dr. HARUO IKEDA for helpful advice.

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