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Two Genes Involved in the Phase-Variable ϕC31 Resistance Mechanism of *Streptomyces coelicolor* A3(2)

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The phage growth limitation (Pgl) system of *Streptomyces coelicolor* confers resistance to ϕC31 and its homoimmune phages. The positions of the *pgl* genes within a 16-kb clone of *S. coelicolor* DNA were defined by subcloning, insertional inactivation, and deletion mapping. Nucleotide sequencing and functional analysis identified two genes, *pglY* and *pglZ*, required for the Pgl$^+$ (phage-resistant) phenotype. *pglY* and *pglZ*, which may be translationally coupled, are predicted to encode proteins with M_rs of 141,000 and 104,000, respectively. Neither protein shows significant similarity to other known proteins, but PglY has a putative ATP/GTP binding motif. The *pglY* and *pglZ* genes are cotranscribed from a single promoter which appears to be constitutive and is not induced by phage infection.

The ϕC31 resistance mechanism of *Streptomyces coelicolor* (termed Pgl, for phage growth limitation) prevents plaque formation on lawns of growing bacteria. Pgl$^-$ strains (sensitive to plaque formation by ϕC31) arise spontaneously from *S. coelicolor* Pgl$^+$ strains at high frequency (10^{-3} to 10^{-4}) and revert to Pgl$^+$ at a similar frequency (9), a situation reminiscent of various kinds of phase variation phenomena such as *Salmonella typhimurium* flagellar antigen variation (reference 17 and references therein), *Neisseria gonorrhoeae* pilin variation (18), expression of the pyelonephritis-associated pili of *Escherichia coli* (4), and expression of outer membrane lipopolysaccharides in *Haemophilus influenzae* (39).

In addition to its phase variation, the Pgl system is interesting because of its novel mechanism (9). ϕC31 propagated on a Pgl$^-$ strain (or the stably ϕC31-sensitive strain *Streptomyces lividans* 66) can adsorb to and lysogenize Pgl$^+$ strains of *S. coelicolor* at the same efficiency as Pgl$^-$ strains. Measurements of the phage lytic cycle using one-step growth curve experiments demonstrated that a single complete cycle of lytic phage development occurs in Pgl$^+$ strains, resulting in a normal burst size of progeny phage. However, the progeny phage are attenuated in their ability to infect further Pgl$^+$ hyphae (although they can infect Pgl$^-$ hyphae). Chinenova et al. (9) proposed that the Pgl system involves at least two components: one that modifies ϕC31 during the initial round of infection and a second that recognizes and attenuates the lytic development of modified ϕC31. The attenuation of modified ϕC31 particles by Pgl$^+$ hyphae does not involve adsorption-inhibition since the frequency of adsorption is the same in Pgl$^+$ and Pgl$^-$ strains (9). Although there is no direct evidence of DNA modification, these observations are consistent with the Pgl system encoding a restriction-modification system in which the restriction endonuclease specifically degrades modified DNA. Restriction systems that act on foreign DNA only when it is methylated occur in several genera (19, 31, 35), including *Streptomyces* (23). However, these systems do not include a modification function, whereas Pgl$^+$ strains appear to modify ϕC31 and then restrict it in subsequent rounds of growth.

The Pgl system therefore represents an inversion of classical restriction-modification systems, whereby progeny phage are modified such that they are no longer restricted. It may represent an adaption to the multicellular growth habit of *Streptomyces* spp., which grow as a branching mycelium. Sacrifice of the hyphal compartment initially infected leads to the inability of the phage to infect other hyphal compartments, thereby protecting the remainder of the mycelium from lysis.

The Pgl system is highly specific for ϕC31 and its homoimmune phages (20), and despite an intensive search, no ϕC31 mutants which are insensitive to the Pgl system have been isolated (20a).

In an attempt to characterize the Pgl system further, we isolated a recombinant plasmid (pIJ5500) carrying a 16-kb fragment of wild-type *S. coelicolor* A3(2) chromosomal DNA that restored a Pgl$^+$ phenotype to four of eight Pgl$^-$ strains tested (20). The strains restored to Pgl$^+$ were defined as Pgl class A strains, and the strains that were not restored to Pgl$^+$ were defined as Pgl class B strains. Here we describe the characterization of the *pgl* genes carried on pIJ5500.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. Bacterial strains are listed in Tables 1 and 2. ϕC31*c1*, a clear-plaque mutant derived from wild-type ϕC31 (21, 26), was used in plaque tests and liquid culture infections. Plaque tests were performed as described previously (15). PMT3009 is an *E. coli* phagemid carrying the *tsr* (thiostrepton resistance) gene (29a).

Media and reagents. For the preparation of total DNA and protoplasts, *Streptomyces* strains were grown in YEME medium (15). For the preparation of RNA, strains were grown in GAE medium (13) supplemented with histidine and uracil; mycelium was harvested by filtration on 0.45-μm-pore-size cellulose acetate filters (Millipore) immediately before RNA isolation. The method of Dowding (10) for one-step growth of phage in *Streptomyces* strains was scaled up to provide enough infected mycelium for isolation of RNA for S1 nuclease mapping.

Cloning methods. DNA manipulations for *E. coli* and *Streptomyces* strains were performed as described by Sambrook et al. (32) and Hopwood et al. (15), respectively. *E. coli* DH5α was used for DNA manipulations. Before the introduction of plasmid DNA from *E. coli* into *S. coelicolor* by transformation, it was passaged through the *dam dcm hsdM E. coli* strain ET12567 (24) to overcome the methyl-specific restriction barrier of *S. coelicolor*. For PMT3009 derivatives, single-stranded DNA was isolated from an F′ derivative of *E. coli* ET12567, using M13K07 helper phage (32).

Construction of subclones. Subclones from the pIJ5500 insert DNA were cloned into the *Bam*HI site of pPM927 (36). The inserts carried by the subclones

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TABLE 1. Derivatives of *S. coelicolor* A3(2) used

John Innes stock code	Genetic markers	Plasmid status	Reference
1147		SCP1$^+$ SCP2$^+$	15
M145		SCP1$^-$ SCP2$^-$	15
J1501	*hisA1 uraA1 strA1 pgl-1*	SCP1$^-$ SCP2$^-$	8
2147	*proA1 argA1 cysD18 pgl-2*	SCP1$^-$ SCP2$^+$	22
1915	*hisA1 uraA1 strA1 pgl-3*	SCP1$^-$ SCP2$^+$	22
JF4Pgl$^-$	*proA1 argA1 uraA1 strA1 act*V-*109 red-60 pgl-6*	SCP1$^-$ SCP2$^-$	11a
TK18Pgl$^-$	*argA1 uraA1 strA1 act* III-*141 red-60 pgl-7*	SCP1$^-$ SCP2$^-$	18b
B135Pgl$^-$	*hisA1 uraA1 strA1 act* V-*235 pgl-8*	SCP1$^-$ SCP2$^+$	18a
B140Pgl$^-$	*hisA1 uraA1 strA1 act* VII-*240 pgl-9*	SCP1$^-$ SCP2$^-$	18a
A3(2)Pgl$^-$	Δ*pglZ10*	SCP1$^+$ SCP2$^+$	9
J1928[a]	*pglY*::*aadA*	SCP1$^-$ SCP2$^-$	This study
J1929[a]	Δ*pglY16*	SCP1$^-$ SCP2$^-$	This study
J1930[a]	Δ*pglY16*	SCP1$^-$ SCP2$^-$	This study
J1931[a]	ORF 4::*hyg* (Pgl$^+$)	SCP1$^-$ SCP2$^-$	This study
J1934[a]	*pglZ*::pIJ5577(*tsr*)	SCP1$^-$ SCP2$^-$	This study

[a] Constructed derivative of M145.

are shown in Fig. 1. The numbers of the restriction sites referred to throughout this report are those designated in this figure. When the restriction enzymes used were not directly compatible with the *Bam*HI cloning site of pPM927, the fragment was cloned into the polylinker of pIJ2925 (16) and reisolated as a *Bgl*II fragment.

Construction of plasmids for gene replacement and insertional inactivation. **(i) pIJ5505.** pIJ5505 was constructed by cloning the 10-kb *Bam*HI fragment (sites 3 to 18) into pIJ2925 digested with *Bam*HI.

(ii) pIJ5513. pIJ5513 was constructed by cloning the 2.9-kb *Pst*I-*Pvu*II fragment (sites 9 to 11) into pIJ2925 digested with *Pst*I and *Sma*I and cloning a kanamycin resistance cassette (as a 1.6-kb *Eco*RI fragment from pUC4KIXX; Pharmacia) into the unique *Eco*RI site of the pIJ2925 polylinker.

(iii) pIJ5815. pIJ5815 was constructed by cloning the 2-kb *Pst*I fragment (sites 1 to 9) carrying part of *pglY* into the *Pst*I site of pIJ2925.

(iv) pIJ5566. A 1.7-kb cassette carrying the *aadA* gene (encoding spectinomycin resistance [30]) was cloned into the *Esp*I site internal to *pglY* (site 7) of pIJ5815. The 3.7-kb *Bgl*II fragment carrying the disrupted *pglY* allele was isolated and cloned into PMT3009 digested with *Bam*HI to create pIJ5566.

(v) pIJ5574. An in-frame deletion in *pglY* was constructed by removing a 543-bp *Sac*II fragment (sites 4 to 8) from pIJ5815 followed by recircularization. The 1.45-kb insert carrying the deletion was removed as a *Bgl*II fragment and cloned into *Bgl*II-digested pGM160 (28) to create pIJ5574.

(vi) pIJ5577. pIJ5577 was constructed by cloning the 2-kb *Pvu*II-*Bsm*I fragment (sites 11 to 12) into *Bam*HI-digested PMT3009. DNA ends were rendered blunt before ligation.

(vii) pIJ5579 and pIJ5580. A 3.2-kb *Sph*I-*Bam*HI fragment (sites 13 to 18) was cloned into PMT3009 to create pIJ5578. A 1.7-kb *Bgl*II cassette carrying the *Streptomyces hygroscopicus* hygromycin resistance gene (*hyg* [41]) was cloned into the unique *Bgl*II site of pIJ5578 in both orientations to create pIJ5579 and pIJ5580.

Sequence analysis. The nucleotide sequence of the 9,461-bp fragment (sites 1 to 16) was determined on both strands by the dideoxy chain termination method of Sanger et al. (33), using Sequenase 7-deaza-GTP (U.S. Biochemical) and TaqTrack (Promega) sequencing kits. The sequence was generated in four parts: the 2- and 0.6-kb fragments (sites 1 to 9 and 15 to 16, respectively) were sequenced by using the universal and custom-made oligonucleotide primers and single-stranded DNA templates derived from M13mp18 and M13mp19. The nucleotide sequences of the 2.7- and 4.5-kb fragments (sites 9 to 11 and 11 to 15) were determined from exonuclease III deletion series (14) of double-stranded templates contained in pBluescriptII vectors (Stratagene), generated by using an Erase-a-Base kit (Promega) according to the manufacturer's instructions. The contiguous sequence was generated by sequencing across the fragment endpoints, using custom-made oligonucleotide primers and pIJ5505 as the template.

Determination of the A3(2)Pgl$^-$ deletion endpoints. The *Pvu*II-*Kpn*I fragment (corresponding to sites 11 to 20) was isolated from a size-selected minilibrary of A3(2)Pgl$^-$ genomic fragments as follows. A3(2)Pgl$^-$ total DNA was digested with *Pvu*II and *Kpn*I and separated on a 0.7% low-melting-point agarose gel. Gel slices corresponding to DNA in the 3- to 5-kb size range were removed, the DNA was isolated, and the fraction containing the desired fragment was identified by Southern blotting using the *Pvu*II-*Bsm*I fragment (sites 11 to 12) as a probe. A minilibrary was made by ligating this fraction with pBluescriptII SK+ digested with *Eco*RV and *Kpn*I and transforming *E. coli* DH5α. Colony hybridization using the same probe identified clones containing the desired fragment, and one such clone was used as a template for sequence analysis.

RNA isolation and high-resolution S1 nuclease mapping. RNA was isolated by the method of Hopwood et al. (15), using DNase I to remove DNA. Forty micrograms of RNA was used in each S1 nuclease protection experiment, with hybridizations carried out in NaTCA buffer (27) at 45°C overnight after denaturation at 65°C for 10 min. Each RNA sample was hybridized with approximately 0.02 pmol of the appropriate probe, equivalent to 20,000 Cerenkov counts min^{-1}.

Nucleotide sequence accession number. The sequence presented in Fig. 2 has been assigned GenBank accession number L37531.

RESULTS

Defining the extent of the *pgl* genes on the cloned DNA. The minimum segment of DNA required to restore a Pgl$^+$ phenotype to Pgl class A strains was determined by subcloning. Seven fragments from the insert of pIJ5500 (Fig. 1) were subcloned into the *Bam*HI site of the pSAM2-derived expression vector pPM927 (36) downstream from the adjacent thiostrepton-inducible promoter, P_{tipA}. pPM927 integrates stably into the *S. coelicolor* chromosome via site-specific recombination at a single attachment site (5, 18a, 25).

None of the seven subclones had any effect on the Pgl phenotype of class A strains when grown in the absence of thiostrepton. However, in the presence of thiostrepton, the two largest subclones, pIJ5518 and pIJ5553, conferred a Pgl$^+$ phenotype on all the class A strains tested [J1501, JF4Pgl$^-$, TK18Pgl$^-$, and A3(2)Pgl$^-$]. The requirement for P_{tipA} induc-

TABLE 2. Derivatives of *E. coli* K-12 used

Strain	Genotype	Reference
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 deoR*	12
ET12567	F$^-$ *dam13*::Tn*9 dcm6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*	24
ET12567F′	ET12567 containing mini-F′ (kanamycin resistant)	1a

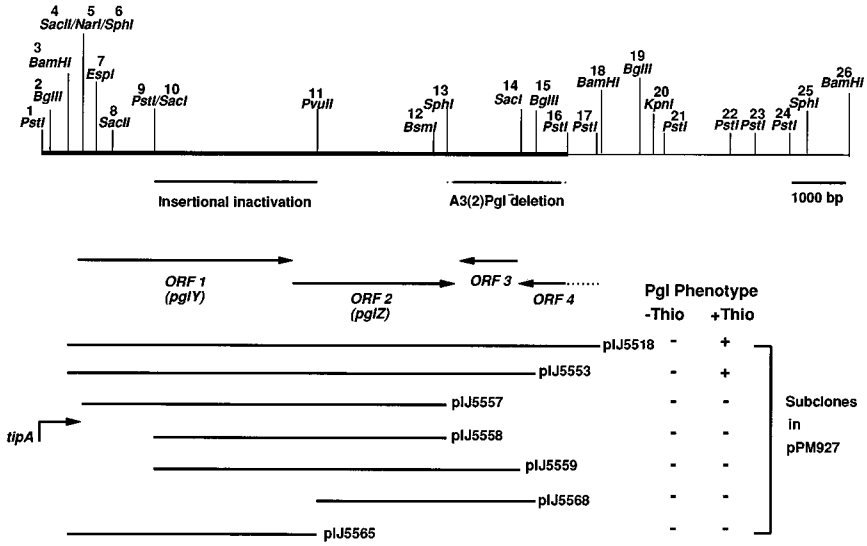

FIG. 1. Restriction map of the 16-kb insert of pIJ5500 showing the 2.9-kb *Pst*I-*Pvu*II fragment that insertionally inactivates the Pgl system and the position of the deletion in the stable Pgl$^-$ strain. The thickened line represents the extent of the sequenced DNA. The subclones made in pPM927 and their abilities to restore a Pgl$^+$ phenotype to Pgl class A strains are illustrated below; the direction of the *tipA* promoter with respect to the inserts was from left to right in all cases. The numbering of restriction sites shown is used throughout the text; not all sites for each restriction enzyme are shown.

tion implied that the *pgl* promoter lies left of *Bam*HI site 3. Neither pIJ5518 nor pIJ5553 had any effect on plaque formation in the Pgl class B strains tested (1915, 2147, B135Pgl$^-$, and B140Pgl$^-$), and there was no evidence that induction of transcription of any of the subclones was toxic to either class A or class B strains.

A 2.9-kb *Pst*I-*Pvu*II fragment (sites 9 to 11) was cloned, along with a kanamycin resistance marker, into the *E. coli* plasmid pIJ2925, and the resulting plasmid (pIJ5513) was introduced into *S. coelicolor* M145 by transformation. Since pIJ2925 (derived from pUC19) cannot replicate in *Streptomyces* spp., selection for kanamycin-resistant colonies identified transformants in which pIJ5513 had integrated into the chromosome by homologous recombination between the 2.9-kb insert and its chromosomal counterpart, as verified by Southern analysis. Plaque tests showed that the transformants were Pgl$^-$, demonstrating that the 2.9-kb *Pst*I-*Pvu*II fragment (sites 9 to 11) is internal to a transcription unit essential for the Pgl$^+$ phenotype (on the basis of the logic of Chater and Bruton [7]).

A stable *S. coelicolor* Pgl$^-$ mutant has been isolated (9). Southern analysis of this strain, A3(2)Pgl$^-$, in the region of the chromosome corresponding to the insert DNA from pIJ5500 revealed a deletion of approximately 2 kb between *Sph*I site 13 and *Pst*I site 16 (20). If there is a causal relationship between the deletion and the stable Pgl$^-$ phenotype, the *pgl* DNA must extend into the deleted region.

Taken together, the position of the deletion, the insertional inactivation, and the subcloning experiments suggested that the *pgl* DNA must extend from left of *Bam*HI site 3 to right of *Sph*I site 13, a minimum of nearly 7 kb.

Sequence analysis. The nucleotide sequence of a 9,461-bp region between *Pst*I sites 1 and 16 was determined (Fig. 2). By using FRAME analysis (2), four potential protein-coding regions (open reading frames [ORFs] 1 to 4) were identified (Fig. 1 and 2); ORF 4 was incompletely sequenced. ORF 1 and ORF 2 would encode unusually large proteins with M_rs of 141,000 and 104,000, respectively. The stop codon of ORF 1 overlaps the start codon of ORF 2, and the stop codon of ORF 4 overlaps the start codon of ORF 3. This arrangement of overlapping stop and start codons has been identified in several systems in which the genes are known to be translationally coupled (29).

The predicted amino acid sequences of the proteins encoded by ORFs 1 to 4 showed no obvious similarity to sequences in the EMBL, SwissProt, Pir, and Nrl3d databases. However, the sequence GXXXXGKS, a potential ATP/GTP binding motif, was identified in the ORF 1 product (box A [38]; the P-loop [34]) as well as the sequence DEA, found in ATP/GTP-hydrolyzing proteins (box B [38]) (Fig. 2).

The orientation of ORFs 1 and 2 is consistent with the need to induce transcription from P_{tipA} in pIJ5518 and pIJ5553 to confer a Pgl$^+$ phenotype on Pgl class A strains. Since the 2.9-kb *Pst*I-*Pvu*II fragment (sites 9 to 11) used for insertional inactivation spans the junction between ORFs 1 and 2, they must be cotranscribed, and ORF 2 (at least) must be necessary for the Pgl$^+$ phenotype.

ORFs 1 and 2 are required for the Pgl$^+$ phenotype. To demonstrate unequivocally that ORFs 1 and 2 are *pgl* genes, defined mutations were constructed in vitro and used to replace the wild-type copy in *S. coelicolor* M145 (Pgl$^+$). Initially, ORF 1 was disrupted by the insertion of a 1.7-kb cassette carrying the *aadA* gene (conferring spectinomycin resistance) into *Esp*I site 7. The strategy for gene replacement is shown in Fig. 3. pIJ5566 was introduced into M145 by transformation, and thiostrepton-resistant (Thio^r) transformants were selected. Southern analysis of four transformants showed that they contained pIJ5566 integrated into the chromosome via homologous recombination on one side or the other of the *aadA* gene (Fig. 3). Plaque tests showed that the two transformants containing pIJ5566 integrated via crossover b were Pgl$^-$, whereas the two containing pIJ5566 integrated via crossover a were Pgl$^+$. This finding was consistent with the fact that crossover a yields a wild-type arrangement of ORFs 1 and 2, whereas crossover b does not (Fig. 3). Earlier subcloning experiments implied that the *pgl* promoter was upstream of *Bam*HI site 3. That crossover a gave a Pgl$^+$ phenotype showed that the *pgl* promoter must lie downstream of *Pst*I site 1, since transcrip-

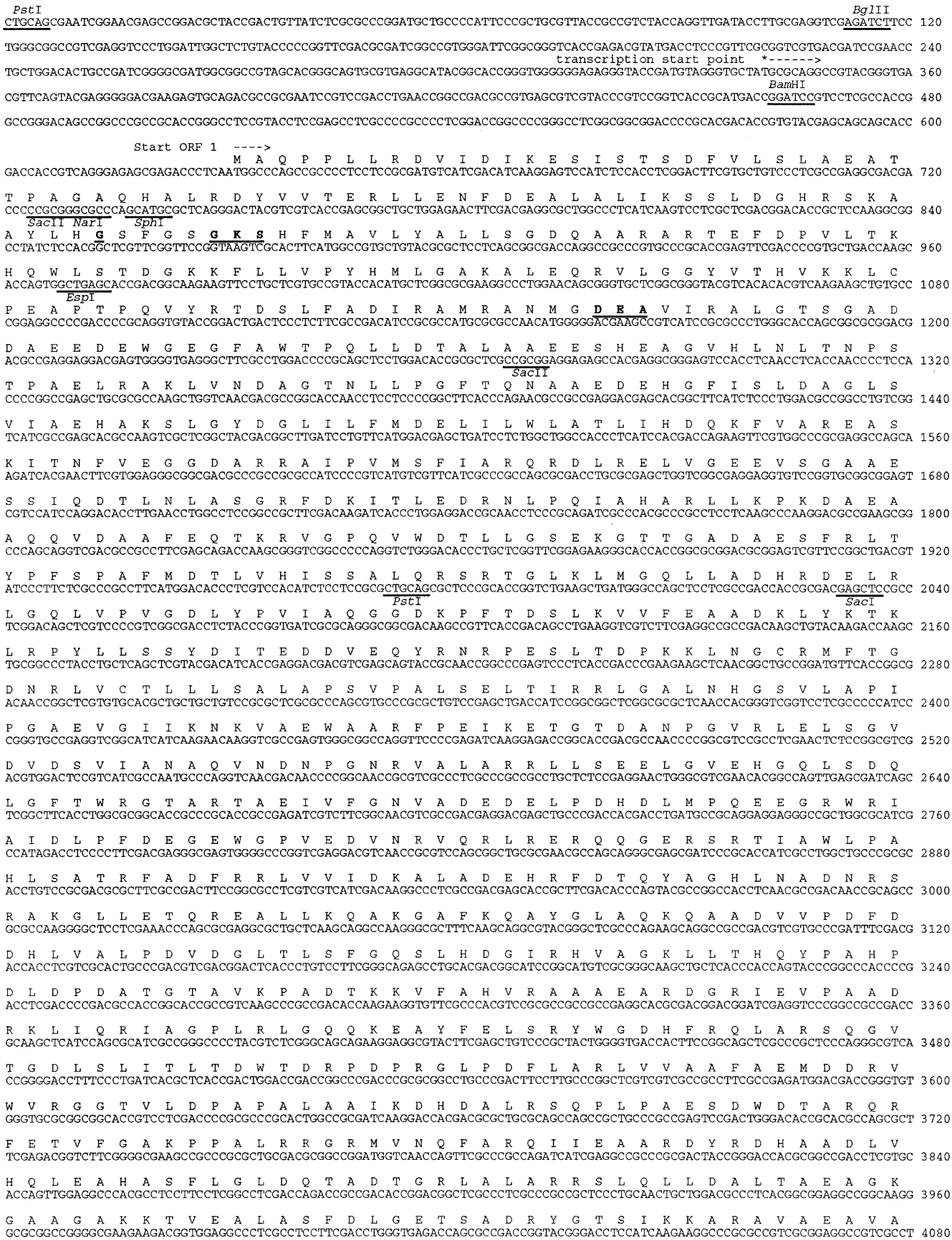

FIG. 2. Nucleotide sequence of a 9,461-bp region (sites 1 to 16) carrying the *pglY* (ORF 1) and *pglZ* (ORF 2) genes. The sequences of both strands are presented in the intergenic region between ORFs 2 and 3, but only the upper strand is presented for the DNA encoding ORFs 1 and 2 and only the lower strand is presented for the DNA encoding ORFs 3 and 4 (which run from right to left). Restriction sites discussed in the text, the transcription start site for the *pglYZ* promoter (*---->), a potential transcriptional terminator between ORFs 2 and 3 (----> <----), and the deletion endpoints (mapped to within two nucleotides) for the Δ*pglZ10* allele in strain A3(2)Pgl$^-$ (*##*) are marked. The amino acids in PglY identified as a potential ATP/GTP binding motif are underlined and in boldface.

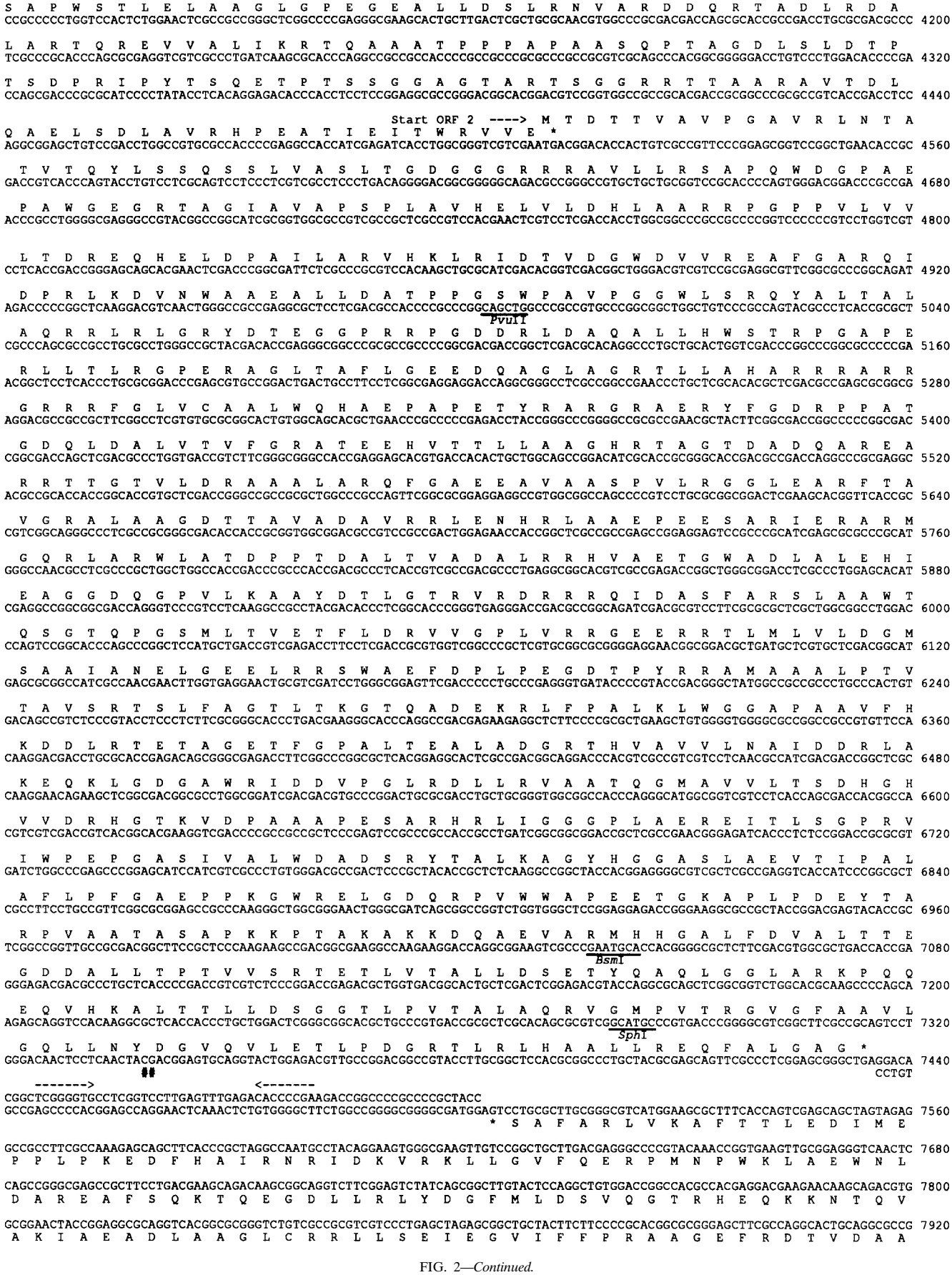

FIG. 2—*Continued.*

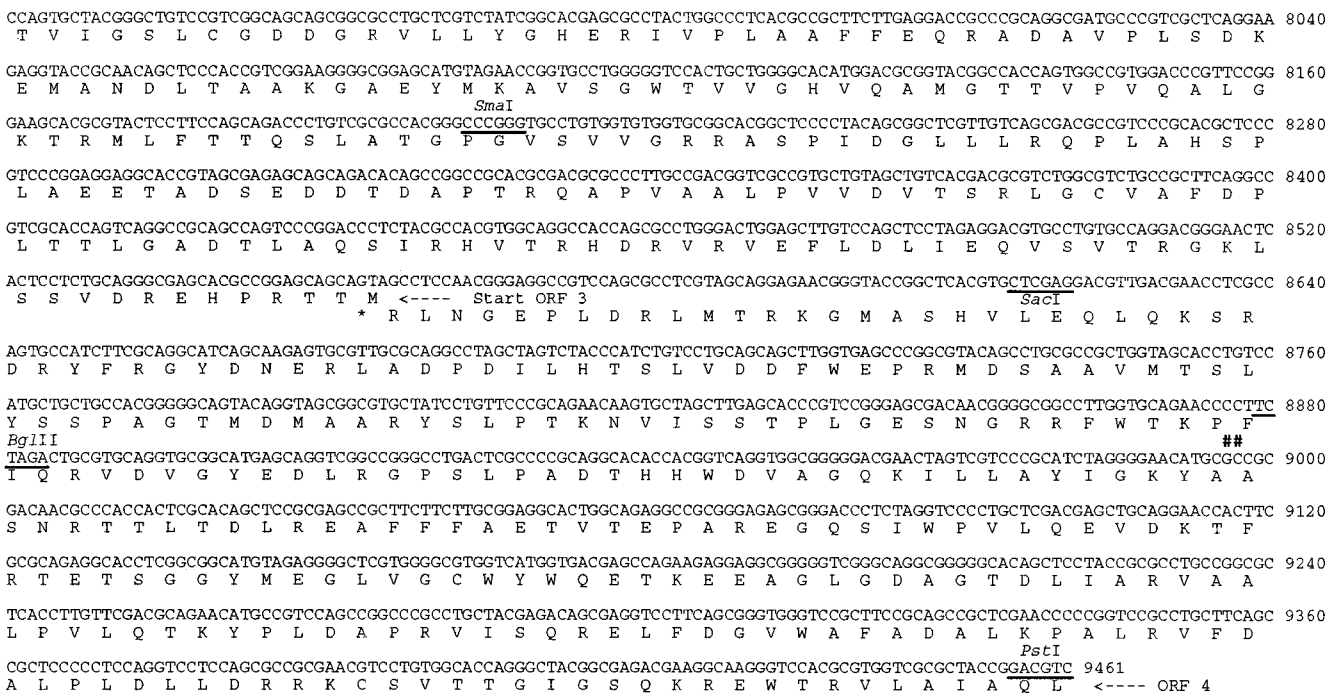

FIG. 2—*Continued.*

tion from vector sequences is prevented by the presence of two copies of the phage fd terminator (Fig. 3).

Spores of J1939 (pIJ5566 integrated via crossover b) were plated on medium containing spectinomycin, and colonies containing potential second crossover events (through crossover a) were identified by screening for loss of Thio^r. One thiostrepton-sensitive (Thio^s), spectinomycin-resistant colony (J1928) was shown by Southern analysis to contain the *aadA* cassette inserted into *Esp*I site 7 (Fig. 3) in the chromosome. Plaque tests showed that J1928 was Pgl$^-$.

To disrupt ORF 1 without the possibility of disrupting expression of ORF 2 through polar effects, an in-frame deletion was constructed in ORF 1 by removing a 546-bp *Sac*II fragment (sites 4 to 8), thus deleting the DNA encoding the potential ATP/GTP binding site of the ORF 1 product. This mutant allele was used to construct pIJ5574 (Fig. 4), based on the temperature-sensitive plasmid pSG5, which is able to replicate in *Streptomyces* spp. at 28°C but not at 39°C (28).

After introduction of pIJ5574 into *S. coelicolor* as an autonomously replicating plasmid (Thio^r), a transformant was grown at 39°C in the presence of thiostrepton to select for integration of the plasmid into the chromosome via homologous recombination. Thio selection was then relaxed while continuing to grow the strains at 39°C, and Thio^s colonies were identified by replica plating. Of 10 nonclonal Thio^s colonies (or colony sectors), 2 (J1929 and J1930) were found by plaque tests to be Pgl$^-$. Southern analysis confirmed that J1929 and J1930 had lost precisely the 564-bp *Sac*II fragment; two Pgl$^+$ Thio^s colonies examined had retained this fragment, presumably because pIJ5574 had integrated and excised through the same interval, thereby giving rise to the parent M145 genotype.

To determine unambiguously if ORF 2 was required for the Pgl$^+$ phenotype of M145, ORF 2 was disrupted by integrating into the chromosome a suicide plasmid (pIJ5577) carrying a 2.05-kb *Pvu*II-*Bsm*I fragment (sites 11 to 12) internal to ORF 2 (Fig. 4). After introduction of pIJ5577 into M145 by transformation, five Thio^r transformants were shown by Southern analysis to contain a disrupted ORF 2, and one was designated J1934. All five transformants were Pgl$^-$.

The plaques formed on the defined ORF 1 and ORF 2 mutants J1928, J1929, J1930, and J1934 were unusually large, like those seen on lawns of the closely related strain *Streptomyces lividans*, which lacks the Pgl system (Fig. 5). These results implied that naturally occurring Pgl$^-$ variants of *S. coelicolor*, such as J1501 (Fig. 5), which support smaller plaques, are not null mutants.

Since ORFs 1 and 2 were both necessary for the Pgl$^+$ phenotype, they were designated *pglY* and *pglZ*, respectively.

ORF 4 is not required for the Pgl$^+$ phenotype. To determine if ORF 4 was required for the Pgl$^+$ phenotype of M145, ORF 4 was insertionally inactivated similarly to *pglY*. A 1.7-kb cassette containing the *hyg* gene was inserted into *Bgl*II site 15 in both orientations to create pIJ5579 and pIJ5580 (Fig. 4). With these plasmids, gene replacement by double crossing-over occurred at high frequency in the primary hygromycin-resistant (Hyg^r) transformants: 4 of 11 M145/pIJ5579 and 6 of 9 M145/pIJ5580 transformants were Hyg^r Thio^s and contained the disrupted ORF 4 allele but no vector sequences, as confirmed by Southern analysis. One such ORF 4 mutant was designated J1931. All the ORF 4 mutants were Pgl$^+$, demonstrating that ORF 4 is not necessary for the Pgl$^+$ phenotype of *S. coelicolor*. Given the potential translational coupling of ORFs 3 and 4, it seems unlikely that ORF 3 would be correctly expressed in these strains, suggesting that ORF 3 is also not required for the Pgl$^+$ phenotype.

A3(2)Pgl$^-$ contains a deletion that inactivates ORF 3, ORF 4, and *pglZ*. To define the endpoints of the Δ*pgl10* deletion precisely, the mutant allele (carried on a 4.6-kb *Pvu*II-*Kpn*I fragment; sites 11 to 20) was cloned (see Materials and Methods). The sequence across the deletion endpoints was determined by using primers based on sequences adjacent to *Sph*I site 13 and *Pst*I site 16. The deletion endpoints were mapped to

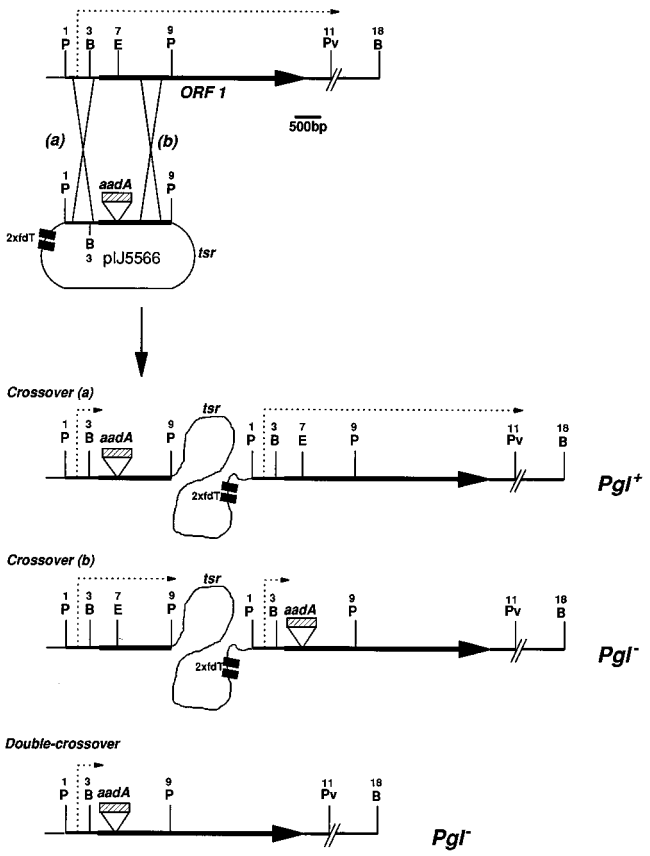

FIG. 3. Homologous recombination between the chromosome and pIJ5566 carrying the *aadA* gene inserted into *Esp*I site 7 within ORF 1. Crossover a leads to a wild-type arrangement of ORFs 1 and 2 and a Pgl$^+$ phenotype, whereas crossover b or the double crossover leads to inactivation and a Pgl$^-$ phenotype. Since insertion via crossover a gives a Pgl$^+$ phenotype, the *pgl* promoter must lie downstream of *Pst*I site 1, as transcription from the vector is blocked by two copies of the phage fd terminator (fdT). P, *Pst*I; B, *Bam*HI; E, *Esp*I; Pv, *Pvu*II.

within two nucleotides (Fig. 2). The deletion is unusual, in that the endpoints do not map to directly repeated DNA sequences (1). The deletion removes 1,657 bp, from nucleotides 7341 to 8998, removing all of ORF 3 and 147 amino acids from the C

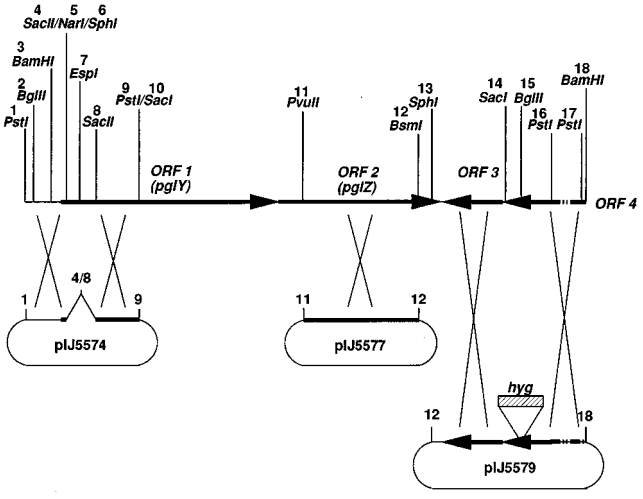

FIG. 4. Strategies for gene replacements of ORFs 1, 2, and 4.

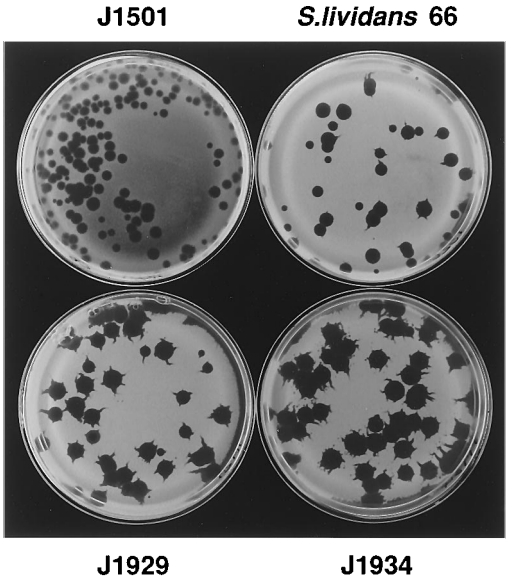

FIG. 5. Phenotypes of constructed *pglY* (J1929) and *pglZ* mutants compared with those of a typical spontaneous *S. coelicolor* Pgl class A mutant (J1501) and the closely related strain *S. lividans* 66, which lacks the Pgl system.

terminus of the ORF 4 product. It also removes 31 amino acids from the C terminus of PglZ, leading to the creation of a predicted fusion protein with at least 155 extra amino acids (the fusion does not reach an in-frame stop codon within the sequenced DNA). It therefore seems likely that the creation of an inactive PglZ fusion protein is responsible for the stable Pgl$^-$ phenotype of strain A3(2)Pgl$^-$.

Transcription of the *pglYZ* operon is not affected by mutations in Pgl class A strains and is not induced by ϕC31 infection. The possibility that components of the Pgl system are induced by ϕC31 infection (20) was investigated by transcriptional analysis of the *pglYZ* genes. With the knowledge that the *pglYZ* promoter lay between *Pst*I site 1 and *Bam*HI site 3, S1 nuclease mapping was used to analyze *pglYZ* transcription in cultures of M145 that had undergone a productive ϕC31 infection in comparison with transcription in an uninfected control culture, using as a probe a 740-bp *Pst*I-*Nar*I fragment (sites 1 to 5) uniquely labelled on the 5′ end of the *Nar*I site. A protected fragment of approximately 400 bases was detected at similar intensities in both infected and uninfected cultures, implying that *pglY* and *pglZ* are transcribed from a single start point and that transcription is not induced by phage infection (Fig. 6a). Comparison of the level of *pglYZ* transcript from uninfected cultures of several Pgl$^+$-Pgl$^-$ pairs, including the defined *pglY* and *pglZ* mutants constructed here, showed that transcription from the *pglYZ* promoter was unaffected by the mutations in four Pgl$^-$ strains [J1501, A3(2)Pgl$^-$, J1929, and J1934] or by inactivation of ORF 4 (J1931) (data not shown).

To identify precisely the transcriptional start point, the S1 nuclease mapping was repeated, using as a probe a 460-bp *Pst*I-*Bam*HI fragment (sites 1 to 3) uniquely labelled on the 5′ end of the *Bam*HI site. Comparison of the S1-protected fragment with the appropriate sequencing ladder pinpointed the transcriptional start point to the T residue at position 341 of the sequence (Fig. 2 and 6b).

The putative −10 and −35 regions of the *pglYZ* promoter show no obvious similarity to the consensus sequence for promoters recognized by RNA polymerase holoenzyme contain-

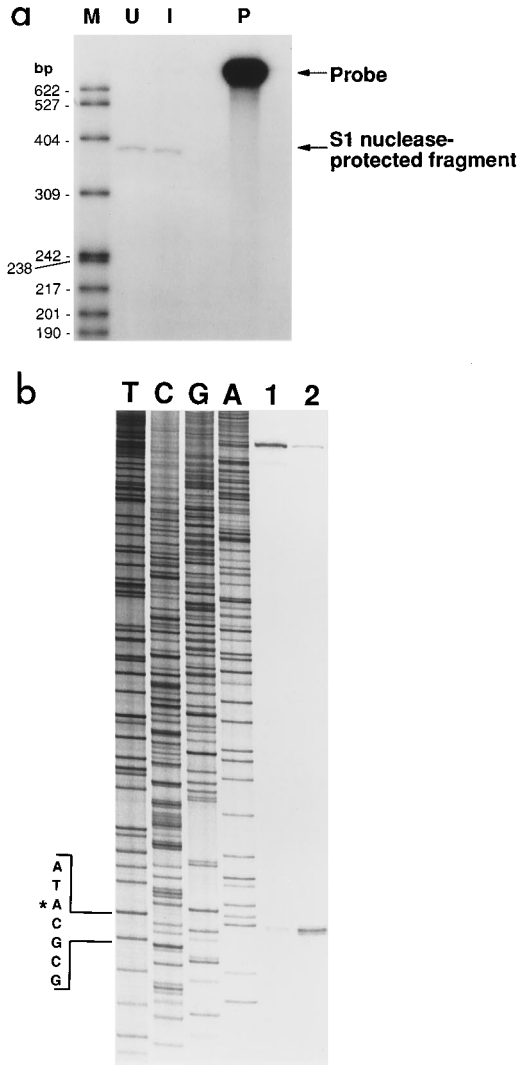

FIG. 6. (a) S1 nuclease mapping of the *pglYZ* transcript in RNA isolated from *S. coelicolor* M145 after a productive ϕC31 infection (I) and from an uninfected control culture (U). Undigested probe (P) and size markers (an *Hpa*II digest of pBR322; M) are also shown. The probe used was a 740-bp *Pst*I-*Nar*I fragment (sites 1 to 5) uniquely labelled on the 5′ end of the *Nar*I site. (b) High-resolution S1 nuclease mapping of the *pglYZ* transcript in RNA isolated from uninfected cultures of J1501 (lane 1) and J1501 carrying the *pglYZ* genes on the single-copy plasmid pIJ5500 (lane 2). The probe used was a 460-bp *Pst*I-*Bam*HI fragment (sites 1 to 3) uniquely labelled on the 5′ end of the *Bam*HI site. The sequencing ladder was generated by dideoxy chain termination using a 17-mer oligonucleotide (5′-GATCCGGTCATGCGGTG-3′) designed to correspond to the first 17 bases of the probe.

ing σ^{hrdB}, the major vegetative σ factor of *S. coelicolor* (6). The most striking feature is a poly(G) tract overlapping the −35 region; similar tracts are present in the −35 region of the *gal P1* and *afsB* promoters (37).

DISCUSSION

The potential translational coupling of *pglY* and *pglZ* may explain why only the plasmid subclones containing both *pglY* and *pglZ* (pIJ5518 and pIJ5553; Fig. 1), not those containing only one of them, restored a Pgl^+ phenotype to the four Pgl class A strains tested. Of these four strains, the *pgl* mutation has been localized only in the case of strain A3(2)Pgl^-; the lesion in *pglZ* in this strain was not complemented by pIJ5559, the subclone containing only *pglZ* (Fig. 1). This may be because pIJ5559 does not express *pglZ* correctly without translation of *pglY*, or alternatively, expression of the wild-type *pglYZ* genes in *cis* may be required for assembly of an active heteromultimer.

The basis of phase variation is understood at the molecular level for a variety of systems (reviewed in reference 11). No DNA rearrangements indicative of DNA inversion at the *pglYZ* locus were detected in Southern blot analysis of Pgl class A strains and their respective parent strains (20), and no sequences suggestive of a phase variation mechanism based on slippage synthesis during DNA replication were identified within the *pglYZ* DNA in the present study.

The predicted amino acid sequences of PglY and PglZ did not reveal significant similarity to other proteins. These observations do not rule out the possibility that these proteins form part of a restriction-modification system, since the sequences of restriction endonucleases are extremely diverse; even type II isoschizomers are often unrelated at the amino acid sequence level (3, 40).

One implication of the model of Chinenova et al. (9) is that Pgl^+ strains do not modify their own DNA, since this would be lethal. Self-protection could result from a selective mechanism of phage DNA modification. Alternatively, one or more components of the Pgl system could be induced in response to ϕC31 infection. Since the *pglYZ* promoter appears to be constitutive, and thiostrepton induction of *pglYZ* transcription is not toxic, self-protection against Pgl cannot be caused by limitation of transcription of *pglYZ* to phage-infected cells. However, the *pglYZ* genes constitute only a part of the Pgl system; they restore a Pgl^+ phenotype to about half of the spontaneous Pgl^- mutants of *S. coelicolor* (Pgl class A strains), and they do not confer phage resistance on the closely related and stably ϕC31-sensitive strain, *S. lividans* (20). The possibility that transcription of other components of the Pgl system (defective in Pgl class B strains) is induced in response to phage infection has yet to be examined.

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