JOURNAL OF BACTERIOLOGY, Nov. 1976, p. 644-650

Vol. 128, No. 2
Printed in U.S.A.

Restriction of a Bacteriophage of *Streptomyces albus* G Involving Endonuclease *Sal*I

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Received for publication 24 August 1976

The bacteriophage Pal6, isolated from soil on *Streptomyces albus* G, was restricted when transferred from an alternative host back to *S. albus* G. Extracted unmodified Pal6 deoxyribonucleic acid was cleaved at a single site by a cell-free extract of *S. albus* G. Fractions cleaving Pal6 deoxyribonucleic acid contained the endonuclease *Sal*I first described by J. Arrand, P. Myers, and R. J. Roberts (unpublished data). A mutant of *S. albus* G was isolated which was defective in both restriction and modification of Pal6. This mutant lacked *Sal*I activity. It is concluded that *Sal*I is the agent of restriction of Pal6 by *S. albus* G.

Many prokaryotes possess enzymes (here called "restriction endonucleases," although often biological restriction activity has not been associated with them) that cleave particular deoxyribonucleic acid (DNA) molecules at specific sites, but in only a few cases has the presence of such an enzyme been correlated with in vivo restriction of phages, plasmids, or DNA introduced by transduction, transformation, or conjugation (1). Partly as a result of this, little is known of the genetic control and biological functions of restriction/modification systems.

The actinomycete *Streptomyces albus* G contains at least two restriction endonucleases (J. Arrand, P. Myers, and R. J. Roberts, personal communication). One of these, *Sal*I, generates cohesive ends during cleavage of DNA and has already proved useful in construction of chimeric plasmids (D. Hamer and C. A. Thomas, Proc. Natl. Acad. Sci. U.S.A., in press; F. Cannon, personal communication). Further study of biological aspects of this enzyme would therefore be of interest in the context of genetic engineering. There are, however, other strong reasons for our interest in this system: first, streptomycetes are often amenable to genetic analysis (6); and second, many streptomycetes produce antibiotics, in at least one case through the agency of self-transmissible plasmids (8, 15). Since both the restriction endonucleases *Eco*RI and *Eco*RII generate DNA fragments with cohesive ends and are plasmid specified (1), it seemed possible that these two features might also coincide in the case of *Sal*I and lead to the identification of a plasmid in *S. albus* G.

In this paper, we describe the isolation of a phage restricted by *S. albus* G and the demonstration that *Sal*I is the agent of this restriction.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Two independent wild-type isolates of *S. albus* were used: strain 1160 was obtained in 1954 by D. A. Hopwood from the Commonwealth Mycological Institute, Kew, England (their strain CMI 52766) and has been maintained in his culture collection since then; and strain 2396 (recently provided by R. J. Roberts) was *S. albus* G of Ghuysen (9). The heat-inducible, lysis-defective lambda lysogen *Escherichia coli* W3110 (containing λ C_I857s7 prophage) was provided by N. Brown.

Enzymes. Deoxyribonuclease I, from beef pancreas, and egg white lysozyme were both from Sigma Chemical Co. Endonuclease *Eco*RI was provided by S. N. Cohen, and *Hin*dIII was from Miles.

Media, buffers, culture methods, and bacteriophage methods. Complete agar medium (5) was used to obtain sporulating growth of *S. albus*, and nutrient broth (Difco) containing 34% (wt/vol) sucrose, 1% $MgCl_2$, and 0.5% glucose (NBS) was used to grow submerged cultures for the preparation of cell-free extracts (7). SM buffer contained 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, 1 mM $MgSO_4$, 100 mM NaCl, and 0.1% gelatin. TE buffer contained 10 mM Tris-hydrochloride, pH 8.0, and 1 mM ethylenediaminetetraacetic acid (EDTA). Extraction buffer contained 10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, and 10 mM 2-mercaptoethanol, sometimes with 10% glycerol added.

General methods for *S. albus* and its phages were as previously described for *S. coelicolor* (2, 5), all incubation being at 30°C. Large-scale confluent top-layer lysates of phage Pal6 for DNA extraction were made in plastic bowls, each equivalent in area to about 50 9-cm petri dishes. To each bowl was added 1,400 ml of nutrient broth agar containing 1.5% agar, 0.5% glucose, and 4 mM $Ca(NO_3)_2$ (2). This was overlaid with 70 ml of soft nutrient broth agar (0.5% agar) containing about 5×10^8 spores of the host strain and enough phages to allow just-confluent lysis of the top layer after overnight incuba-

tion. (For strain 2396 this required 5×10^5 plaque-forming units, and for strain 1160, on which the plaques were larger, it required 5×10^4 plaque-forming units.) The top layer was scraped off as a slurry in 100 ml of nutrient broth and left for 2 h, and the agar was removed by low-speed centrifugation. The agar was reextracted for 2 h with 75 ml of nutrient broth and again removed by centrifugation. The two supernatants were combined. The phages were then pelleted by centrifugation at 4°C (22,500 rpm, 90 min, 30 rotor of a Beckman Spinco L-50 centrifuge or 17,000 rpm, 16 h, JA20 rotor of a Beckman J21 centrifuge), gently resuspended in SM buffer (1/100 of the original volume), and clarified by low-speed centrifugation. CsCl was added (to a density of 1.55 g/ml), and the phage was banded by centrifugation at 33,000 rpm for 21 h in the 40 rotor of a Beckman Spinco L-50 centrifuge. The phage band was separated by collecting drops from the bottom of the centrifuge tube and dialyzed against SM buffer. Bacteriophage λ was induced from *E. coli* W3110 by shifting an aerated exponentially growing nutrient broth culture from 28 to 37°C at extinction at 540 nm of about 0.3 and continuing incubation for a further 3 to 5 h before harvesting the cells by centrifugation. The cells were resuspended in SM buffer containing deoxyribonuclease (10 μg/ml) and lysed with $CHCl_3$ (2%, vol/vol). Free λ phages were concentrated and purified as for Pal6, except that the CsCl was added up to a density of 1.50 g/ml.

DNA extraction. Phage preparations (absorbancy at 260 nm adjusted to between 2 and 10) were extracted three times with equal volumes of TE buffer-saturated redistilled phenol. The DNA solutions obtained were dialyzed extensively against TE buffer.

Endonuclease extraction. *S. albus* mycelium was harvested by centrifugation from 42-h cultures grown at 30°C in a rotary shaker at 150 rpm in 2-liter Erlenmeyer flasks containing 500 ml of NBS. The mycelium was washed once with 10% glycerol and stored frozen at −20°C. For extraction, 2 to 4 g (packed wet weight) of mycelium was suspended (in the ratio 1:2, wt/vol) in extraction buffer containing lysozyme (2.5 mg/ml) and incubated at 37°C for 15 min. After the suspension was cooled in ice, the mycelium was lysed by ultrasonic treatment for long enough (usually about 30 s on the lowest setting of a Soniprobe type 7530A; Dawe Instruments, London, U.K.) to reduce the initially high viscosity before high-speed centrifugation (35,000 rpm, 2 h, 40 rotor of a Beckman Spinco L-50 centrifuge). Solid NaCl was added to the supernatant to 1.0 M, and about 2.5 ml was applied to a Bio-Gel A-5m column (Bio-Rad) equilibrated with extraction buffer containing 1.0 M NaCl. Fractions (40 drops) were eluted with the same buffer and stored at 4°C.

Endonuclease digests. Digests, final volume 50 μl, contained 0.2 to 1.0 μg of DNA and appropriate amounts of restriction endonucleases in 6 mM Tris-hydrochloride, pH 7.9, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 mM NaCl (usually contributed by the use of 5-μl aliquots of fractions containing 1.0 M NaCl). Incubation was at 37°C for 1 to 16 h. Digestion was stopped by incubation at 60°C for 10 min; the tubes were then immediately placed in ice.

Agarose slab gel electrophoresis. The apparatus was from Cold Spring Harbor Laboratory and was used as previously described (12). All gels contained 1% (wt/vol) agarose (Sigma or Seakem), with a supporting layer of 1.4% agarose in the lower 10% of the gel. The running buffer and gels contained 40 mM Tris-acetate, pH 7.9, 5 mM sodium acetate, 1 mM EDTA, and ethidium bromide (0.5 μg/ml). Electrophoresis varied from 16 h at 8 mA to 2.25 h at 75 mA. DNA bands were photographed through a red filter on Polaroid 55 film, under excitation at 365 nm.

RESULTS

Isolation of a phage for *S. albus* G. A number of bacteriophages previously isolated on other streptomycetes were tested for plaque-forming ability on strain 2396. In no case were plaques obtained. This may have been due to unknown host range effects or to massive restriction. The phages tested were: virulent phages VP8, VP11, VP12, and VP13 (2; J. E. Dowding, Ph.D. thesis, University of East Anglia, Norwich, U.K., 1972), φ448 (16), and f6 (provided by S. T. Williams and K. I. Sykes, Hartley Botanical Laboratories, University of Liverpool, Liverpool, U.K.); and temperate phages VP5 (3) and φC31 (10).

To obtain phages that did form plaques on strain 2396, we used a simple enrichment technique (2) on various soil samples. One of the phages obtained was Pal6, with which this paper is concerned.

Restriction of Pal6 by strain 2396. Pal6 was tested on several *S. albus* wild types from the culture collection of D. A. Hopwood in order to find a nonrestricting, nonmodifying alternative host. Numerous large clear plaques were obtained on strain 1160, and tests were made for restriction by strain 2396 of Pal6 (1160), that is, Pal6 last propagated on strain 1160 (Table 1). There were about one million-fold fewer plaques on 2396 than on 1160 with Pal6 (1160),

TABLE 1. *Plaque-forming abilities of various Pal6 preparations on two strains of Streptomyces albus*

Previous hosts of Pal6 since isolation on 2396[a]	Relative EOP on 2396[b]
1160	1×10^{-6}
1160, 2396	5×10^{-1}
1160, 2396, 1160	1×10^{-2}
1160, 2396, 1160, 2396	3×10^{-1}
1160, 2396, 1160, 2396, 1160	1×10^{-2}

[a] Given in sequence, so that the last host is given last.

[b] The EOP on strain 1160 was always higher than on 2396 and is therefore set at 1 in all these experiments.

whereas Pal6 (2396) gave only three times fewer on 2396 than on 1160. To determine whether this change in EOP (efficiency of plating) was due to restriction and not, for example, to the selection of host range mutants, we replated Pal6 (2396) on 1160 and then back on the 2396. The EOP was about 10^{-2} instead of the expected 10^{-6} (relative to that on 1160). Further passages of phage from these plaques through 1160 and back on to 2396 always gave an EOP of 10^{-2}. This observation of a change from an EOP of 10^{-6} to one of 10^{-2} was repeated with three preparations of Pal6 (1160) soaked out from single plaques on 2396. Clearly, mutant phages were being selected that had a higher EOP on 2396. We shall use the term Pal6 *sag* (for *S. albus* G host range mutation) for such mutants, of which only one was used in the experiments that follow.

Cell-free extracts of strain 2396 contain endonuclease *Sal*I. Fractions of a cell-free extract of 2396 obtained after Bio-Gel A-5m chromatography were incubated with λ DNA, and the digestion products were analyzed. A broad peak of endonuclease activity was observed. Using active fractions, either alone or in combination with *Eco*RI, two closely linked cleavage sites were apparent at about 69% on the λ map, both in *Eco*RI fragment 2 of λ DNA (4). This coincided with the observations of J. Arrand, P. Myers, and R. J. Roberts (personal communication) on the endonuclease *Sal*I obtained by them from strain 2396. We could detect no other restriction endonuclease activity in our extracts, although a second enzyme with many more cleavage sites in λ DNA has been detected in 2396 grown under certain cultural conditions (Arrand et al., personal communication).

A fraction containing *Sal*I cleaves unmodified Pal6 DNA. DNA prepared from Pal6 (1160), Pal6 *sag* (1160), or Pal6 *sag* (2396) was digested with an active 2396 fraction from Bio-Gel chromatography and electrophoresed on agarose gels. We initially detected no cleavage of any of these DNAs. However, a very small number of cleavages generating either very large or very small fragments would have been relatively difficult to detect, since fragments of greater than about 2×10^7 g/mol are very poorly resolved on 1% gels, whereas small fragments would have been less easily visualized because of their proportionately reduced binding of ethidium bromide. We therefore examined the various Pal6 DNAs in double digests with an active fraction of a 2396 extract and *Eco*RI (Fig. 1).

All of the DNAs gave a characteristic, surprisingly complex, and apparently invariant banding pattern after digestion with *Eco*RI alone. The pattern was unchanged after digestion for longer times and with more *Eco*RI and also with different Pal6 DNA preparations, and electrophoresis of *Eco*RI digests of mixtures of λ DNA and Pal6 DNA gave the expected superimposition of the patterns obtained with sepa-

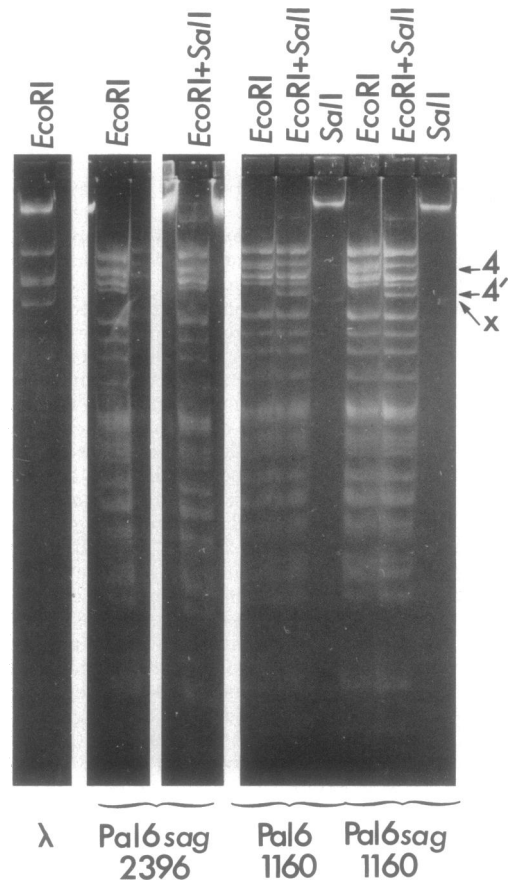

FIG. 1. *Electrophoresis of various Pal6 DNA species digested by restriction endonucleases. The endonucleases used are indicated above each track; SalI was a crude extract of strain 2396 (see text). The type of DNA used (including the host on which the Pal6 was propagated) is given below each track. The arrow 4 indicates the position of EcoRI band 4, which is cleaved to give a new fragment (arrow 4') using DNA from phage last propagated on strain 1160. A small amount of 4' was also seen with DNA from Pal6 sag (2396). The arrow x indicates a faintly visible band generated by SalI alone, which is more clearly seen in the microdensitometer scans shown in Fig. 3. In these experiments, λ DNA was digested with EcoRI at the same time and under the same conditions and was run on the same gels; the track shown here was from the same gel as the six right-hand tracks.*

rate *Eco*RI digests of the two kinds of DNA. The pattern observed with Pal6 DNA after *Eco*RI digestion must therefore show the products of complete and not partial *Eco*RI digestion. Analysis of this pattern by microdensitometry was used to determine approximate molar ratios of bands and to give them identifying numbers (1 through 42; Fig. 2). Molecular weights were allocated to fragments by comparing their mobilities with those of standards run on the same gels (we used λ DNA digested with *Eco*RI or *Hin*dIII (4, 11); a straight line was obtained by plotting the logarithm of molecular weight against distance migrated for standard fragments of molecular weight less than 4.67×10^5). The summed molecular weight of all the Pal6 DNA fragments was 6.6×10^7 (Table 2). This figure may be unreliable as an estimate of the molecular weight of Pal6 DNA for several reasons: the method of calculation takes no account of probable differences in base composition between Pal6 and λ DNA; low-molecular-weight standards were not available; and precise estimates of the numbers of fragment species comprising each band were difficult from fragment 14 onwards. In what follows, we express the size of fragments as a percentage of the summed molecular weight.

In the double digests, *Eco*RI fragment 4 (5.98%) disappeared when the DNA was from Pal6 (1160) or Pal6 *sag* (1160), and approximately equimolar amounts of a new band (4.89%) appeared. No obvious band or bands corresponding to the remaining 1.09% were seen, though there was a suggestion of increased fluorescence in the band comprising fragment 37 and 38 (0.68%). With DNA from Pal6 *sag* (2396) there was no obvious reduction in the level of fragment 4, although a faint trace of the new 4.89% band (in 7- to 10-fold-lower molar amounts) was seen. No differences could be detected between the double-digest banding patterns of Pal6 *sag* (1160) and Pal6 (1160).

In single digests of various Pal6 DNAs with *Sal*I-containing fractions (Fig. 1), in which more DNA was used than in our initial experiments, we observed a band corresponding to 4.70% of the total DNA in addition to the major slowly migrating band. This band migrated slightly, but definitely, faster than the new band seen in the double digests of Fig. 1. Microdensitometry (Fig. 3) showed that it was present in approximately equimolar amounts to the main band when DNA from Pal6 (1160) or Pal6 *sag* (1160) was the substrate but in about 10-fold lower molar amounts when the DNA was from Pal6 *sag* (2396).

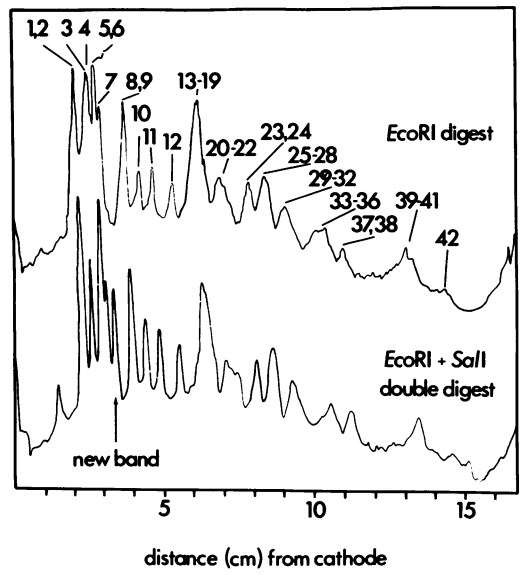

FIG. 2. *Microdensitometer analysis of electrophoretic patterns of Pal6 sag (1160) DNA digested by restriction endonucleases (from the gel shown in Fig. 1). SalI was a crude extract of strain 2396 (see text). Numbers designate fragment species, which are further analyzed in Table 2.*

Combining information from the single and double digests, we conclude that there is probably a single *Sal*I cleavage site in unmodified Pal6 DNA, situated 4.70% along the length of the molecule. The nearest *Eco*RI cleavage sites on either side of this site were at points 3.61 and 9.59% along the molecule (Fig. 4).

Isolation and analysis of a restriction-deficient mutant of 2396. Using increased sensitivity to Pal6 *sag* (1160) spread on nutrient agar plates as a test for restriction deficiency (K. F. Chater, unpublished data), we isolated a 2396 mutant defective in restriction, after ultraviolet irradiation of a spore suspension to 0.01% survival. The starting strain for this experiment was an isoleucine-plus-valine auxotroph (*ilv-1*), and the restriction-defective (R^-) mutant described here had the Ilv^- phenotype. Plating tests with Pal6 *sag* (1160) and Pal6 *sag* (2396) (Table 3) confirmed that the EOP did not depend on the previous host of the phage. Pal6 *sag* soaked out from plaques on the mutant was restricted by 2396 (Table 3) by about the same factor as Pal6 *sag* (1160) [note that the figure for the EOP of Pal6 *sag* (1160) on 2396 in Table 3 is somewhat lower than that in Table 1 because of the use of the *ilv-1* strain]; thus the mutant was also modification deficient (M^-).

It was also found (Table 3) that the EOP of

TABLE 2. *Estimated sizes of EcoRI cleavage products of Pal6 DNA*

*Eco*RI fragment no.	Estimated molecular mass (megadaltons)[a]	% of Pal6 DNA	*Eco*RI fragment no.	Estimated molecular mass (megadaltons)[a]	% of Pal6 DNA
1	4.70	7.12	22	1.15	1.74
2	4.55	6.89	23	0.98	1.48
3	4.10	6.21	24	0.98	1.48
4	3.95	5.98	25	0.85	1.29
5	3.65	5.53	26	0.85	1.29
6	3.65	5.53	27	0.83	1.26
7	3.50	5.30	28	0.83	1.26
8	2.80	4.24	29	0.72	1.09
9	2.80	4.24	30	0.72	1.09
10	2.50	3.79	31	0.69	1.05
11	2.20	3.33	32	0.66	1.00
12	1.65	2.50	33	0.57	0.86
13	1.50	2.27	34	0.52	0.79
14	1.50	2.27	35	0.52	0.79
15	1.50	2.27	36	0.52	0.79
16	1.45	2.20	37	0.45	0.68
17	1.45	2.20	38	0.45	0.68
18	1.45	2.20	39	0.25	0.38
19	1.40	2.12	40	0.25	0.38
20	1.25	1.89	41	0.25	0.38
21	1.22	1.85	42	0.20	0.30

[a] Sum of all molecular masses, 66.01 megadaltons.

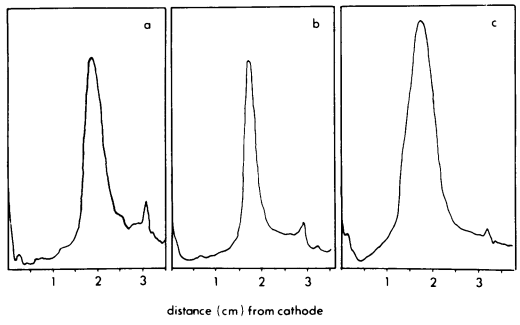

FIG. 3. *Microdensitometer analysis of electrophoretic patterns of various Pal6 DNA preparations digested with an extract of strain 2396. (a) Pal6 (1160) DNA; (b) Pal6 sag (1160) DNA; (c) Pal6 sag (2396) DNA. The mobility of the minor band suggests that it constitutes 4.7% of the total molecular weight of Pal6 DNA, and the areas under the peaks give an approximately 1:1 molar ratio for (a) and (b), whereas for (c) there is about a 10-fold molar excess of the major peak.*

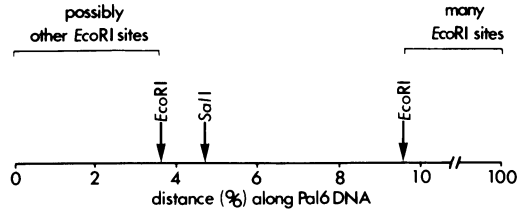

FIG. 4. *Endonuclease cleavage sites on Pal6 DNA.*

Pal6 (1160) on the mutant was higher than that on restriction-proficient 2396, by a factor of about 40-fold. Since this was similar to the factor by which Pal6 *sag* (1160) was restricted by 2396, and which was lost in the mutant, we concluded that the EOP of about 10^{-6} of Pal6 (1160) on 2396 compared with that on 1160 was due to two effects: restriction by the system accounted for about a 40-fold reduction in the EOP, and some other unknown factor inde-

TABLE 3. *Plaque-forming abilities of various Pal6 preparations on a restriction-deficient (R^-) mutant of 2396*

Pal6 prepn[a]	Relative EOP[b]	
	Strain 2396	Strain 2396 R^-[c]
Pal6 *sag*[d] (1160)	7.0×10^{-3}[e]	7.5×10^{-1}
Pal6 *sag*[d] (2396)	4.5×10^{-1}[e]	7.3×10^{-1}
Pal6 *sag*[d] (2396 R^-)	2.4×10^{-2}	NT[f]
Pal6 (1160)	6.2×10^{-7}	2.5×10^{-5}

[a] Last host of Pal6 indicated in parentheses.

[b] For each experiment, the EOP with strain 1160 was defined as 1.

[c] This restriction-deficient (R^-) mutant was isolated from an isoleucine-plus-valine auxotroph (*ilv-1*).

[d] Pal6 *sag* is a mutant of Pal6 having a higher EOP than Pal6 on strain 2396.

[e] These figures were obtained with the *ilv-1* parent strain of the restriction-deficient mutant.

[f] Not tested.

pendent of that restriction accounted for the remaining factor of 10^{-4} to 10^{-5}.

A cell-free extract of this mutant fractionated as described in Materials and Methods contained no detectable *Sal*I activity in any fraction when tested in 16-h digests with λ DNA, either in single digests or in double digests in which *Eco*RI was also present. No fraction inhibited either *Eco*RI or active *Sal*I, so that the negative results with fractions from the mutant were not due to inhibition of endonuclease activity. We concluded that the mutant did not contain *Sal*I. Finally, to eliminate the remote possibility that an enzyme other than *Sal*I was present that could cleave Pal6 DNA, double digests of alternate fractions with *Eco*RI were incubated with Pal6 *sag* (1160) DNA for 16 h. No fraction gave rise to cleavage of *Eco*RI/Pal6 band 4.

DISCUSSION

An extensive 1969 review on bacteriophages of actinomycetes (14) referred to only one case of what might have been restriction; however, the original data (13) were interpreted in terms of some kind of host range mutation and do not seem to have been due to restriction. Restriction of Pal6 by *S. albus* G is thus the first well-documented description of restriction in *Streptomyces*.

Strong circumstantial evidence suggests that the agent of restriction is the endonuclease *Sal*I, which cleaves unmodified Pal6 DNA at a single site about 4.7% along the length of the molecule. This cleavage occurs only with a small fraction (about 10%) of DNA prepared from Pal6 grown on 2396, presumably because most of the DNA is modified. A mutant of strain 2396 that does not restrict Pal6 contains no detectable *Sal*I activity.

The level of restriction apparently due to *Sal*I activity is about 10^{-2}. However, an EOP of about 10^{-6} was observed when Pal6 that had never (knowingly) been subjected to restriction by 2396 was transferred from the alternative host 1160 to 2396. For two reasons, we believe that the extra factor of 10^{-4} over and above the subsequently observed level of restriction is not related to *Sal*I-mediated restriction: (i) there was no detectable difference in the *Sal*I cleavage patterns of DNA isolated from the two kinds of unmodified phage (Fig. 1), and (ii) a 2396 mutant defective in the 10^{-2} level of Pal6 restriction in vivo and in *Sal*I activity in vitro still gave an EOP of only 2.5×10^{-5} for unmodified Pal6 having an EOP of 6.2×10^{-7} on the 2396 wild-type parent strain. We have not tried to find out whether a second restriction system or some different mechanism is involved; however, it is clear that the rare plaques obtained with Pal6 (1160) on strain 2396 are of mutant phages that have become insensitive to whatever mechanism is involved. The origin of this phenomenon is particularly mysterious since Pal6 was originally isolated on strain 2396. However, the plaques first obtained when isolating Pal6 from soil were minute, and when phages were soaked out from them no plaques could be obtained with the suspension on strain 2396, although some were obtained in a spot test with strain 1160; it was from these that our Pal6 stock was derived. There is reason to believe that Pal6 (1160) does infect *S. albus* G at a much higher level than is indicated by the EOPs given in Tables 1 and 2, but that most of the infections result in plaques so small as normally to be invisible (K. F. Chater, unpublished data). The original plaques were probably of this kind, but because of some aspect of the conditions prevailing at the time of isolation they were just visible. The Pal6 *sag* mutant might not have been selected at this stage because the Pal6 population had never been high enough for it to have occurred with significant probability.

The small amount of *Sal*I cleavage of DNA from Pal6 propagated on strain 2396 (Fig. 1 and 3) may result from failure of the modifying host to modify every phage DNA molecule, from a failure of completely modified DNA to be completely resistant to *Sal*I cleavage, or from some activity present in the extracts used that can strip the modified DNA of its modification.

ACKNOWLEDGMENTS

The discussions with Richard J. Roberts and the technical instruction by Phyllis Myers received by K.F.C. during a short stay in Rich Roberts's laboratory at Cold Spring Harbor were a sine qua non of this work. That visit was made possible by the generosity both of the Cold Spring Harbor Laboratories and of the British Agricultural Research Council. We also acknowledge invaluable discussions with John Arrand; the encouragement of David Hopwood; and the work of L. S. Clarke and S. Frey in designing a black-light box and photographing many gels with its aid.

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