A comparison of DNA cleavage by the restriction enzymes SalPI and PstI

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ABSTRACT

Methods for obtaining highly active, exonuclease-free, stable preparations of the Streptomyces albus P restriction enzyme SalPI are described. SalPI and its isoschizomer PstI (from the taxonomically distant Providencia stuartii 164) both cleave their recognition sequence (5'-CTGCAG-3') to generate fragments terminating in tetranucleotide 3' extensions whose sequence is 5'-TGCA-3'. Bacteriophage R4G2 DNA, protected against SalPI cleavage by pregrowth on S. albus P, is also protected against PstI cleavage; and total DNA of both S. albus P and P. stuartii 164 is resistant to cleavage by both enzymes.

INTRODUCTION

The site-specific endodeoxyribonuclease SalPI[2] is implicated in restriction of bacteriophages.[3] The producing organism, Streptomyces albus P, also contains a modification system which protects SalPI target sites against cleavage by SalPI.[3]

SalPI recognises the same hexanucleotide sequence as the endonuclease PstI isolated from Providencia stuartii 164.[4] The recognition site (5'-CTGCAG-3') is cleaved by PstI to give tetranucleotide 3' extensions whose sequence is TGCA.[5] PstI is a particularly useful enzyme for DNA cloning because the addition of oligo-dG residues to the 3'-termini of PstI-generated DNA fragments and of oligo-dC residues to the termini of any other DNA molecules, provides a selective system for generating hybrid DNA molecules which can subsequently be cleaved at the regenerated PstI sites.[6] In this paper we further examine the similarity of SalPI and PstI.

MATERIALS AND METHODS

DNA. Preparation of DNA was as previously described for phages λ CI857 Sam7[7] and R4G2[3] and for phage ØX174 am3 cs70 replicative form (RFI) DNA.[8] Plasmids pBR322 and pAT153 were gifts of Dr A. G. Hepburn and ØX174 am3 cs70 (+) strand and (-) strand DNAs were gifts of Dr B. G. Barrell. Total bacterial DNA was isolated by a procedure devised by T. Kieser (to be published), the essential steps of which were: lysis of cells by lysozyme-pronase-SDS treatment; two phenol plus chloroform extractions; two chloroform extractions; RNase treatment; precipitation by polyethylene glycol; and precipitation with ethanol. The yields were 5 mg of Providencia stuartii 164 DNA and 2.5 mg Streptomyces albus P DNA from 1 g wet weight frozen cells.

Enzymes and reagents. Sources of enzymes were as follows: PstI, EcoRI and E. coli DNA polymerase I fragment A (Klenow enzyme), Boehringer Mannheim; HhaI, Bethesda Research Laboratories Inc.; T4 DNA polymerase, Miles Laboratories; lysozyme (3 x crystallized) and RNase A (5 x crystallized), Sigma. T4 DNA ligase was prepared as in (9) from a heat-induced culture lysogenic for λ NM989, which contains a cloned T4 DNA ligase gene.[10] The lysogen was kindly provided by Dr K. Murray. SalGI was prepared by the polyethyleneimine (procedure B) heparin-Biogel method,[11] peak activity eluting at 0.45M NaCl. SalPI was prepared as described below. Sources of reagents used in DNA sequencing were as in (12).

Restriction enzyme digestions. These were done at 37°C in 10mM tris HCl pH 7.4, 10mM $MgCl_2$, 10mM 2-mercaptoethanol, 50mM NaCl (100mM NaCl for SalGI). Analysis of digests was by electrophoresis in vertical or horizontal 20 cm x 20 cm x 0.3 cm, 1% agarose (Miles Laboratories, LE grade) gels in a buffer containing 40mM tris acetate pH 7.9, 20mM sodium acetate, 1mM EDTA, at 70v for 16 h, followed by staining in ethidium bromide (0.5 μg/ml in water for 30 min) and illumination with a C61 transilluminator (U.V. Products, San Gabriel, California). By definition, one enzyme unit completely digested 1 μg λ DNA in a volume of 20 μl in 1 h.

Ligation. Samples of DNA (0.5 μg) completely digested by restriction

endonuclease were incubated with T4 DNA ligase (4 units) in 66mM tris HCl pH 7.5, 1mM EDTA, 10mM $MgCl_2$, 10mM 2-mercaptoethanol, 0.1mM ATP (final volume 60 µl) at 0°C for 1 h and then 12°C for 1 h. The reaction was terminated by heating at 70°C for 10 min.

Preparation of SalPI. Unless stated otherwise, all stages were at 4°C.

(a) Using heparin-Biogel chromatography.[11] Typically, 25 g (wet weight) S. albus P cells were suspended in 50 ml extraction buffer (100mM tris HCl pH 8.0, 10mM 2-mercaptoethanol, 2mM EDTA) containing lysozyme (2.5 mg/ml) and incubated at 30°C for 30 min with occasional stirring. After cooling in ice the suspension was sonicated (2 x 30 sec., setting 3, Dawes Soniprobe) to complete lysis and to reduce the viscosity. Subsequent processing [polyethyleneimine precipitation of DNA, ammonium sulphate precipitation, redissolving the pellet in column buffer (20mM tris HCl pH 7.5, 7mM 2-mercaptoethanol, 0.5mM EDTA), heparin-Biogel chromatography] was as in method B of Bickle et al.,[11] (method A, in which co-precipitation of enzyme and DNA by polyethyleneimine is used,[11] yielded no SalPI activity). SalPI activity eluted at about 0.3M NaCl and usually contained traces of non-specific deoxyribonuclease activity. Peak fractions were pooled, dialysed against column buffer, and chromatographed on a 5 ml column of DEAE cellulose (Whatman, DE52, prepared as in (13); column height : diameter ratio 5 : 1). SalPI eluted at about 0.25M NaCl in a 0 - 1.0M NaCl gradient. Peak fractions were pooled and dialysed against column buffer containing 50% glycerol, and stored at -20°C.

(b) Using phosphocellulose and hydroxylapatite chromatatography.[14] After initial cell lysis as in the heparin-Biogel procedure, phosphocellulose and hydroxylapatite chromatography were done as in (14). The SalPI activity peak eluted from phosphocellulose at about 0.35M NaCl and from hydroxylapatite at about 0.25M NaCl. Similar values were reported for elution of PstI.[14] Peak fractions were dialysed against column buffer plus 50% glycerol and stored at -20°C.

Determination of the SalPI cleavage site. The phosphodiester bond cleaved in each strand of ØX174 RFI DNA at its single SalPI (≡ PstI) target site was identified in relation to the DNA sequence determined by the chain

termination method.[15] The primer fragments used were HhaI fragment 4 (for use with (+) strand DNA as template) and HhaI fragment 15 (with (-) strand DNA as template), purified electrophoretically.[16] These fragments map on either side of HhaI fragment 8, which contains the SalPI/PstI target site.[17] In experiments using HhaI fragment 4, the primer fragment was removed by cleavage with HhaI before analysis of the reaction products on thin acrylamide-urea gels.[18]

For each primer-template combination, in addition to the four sequencing reactions, a fifth reaction was performed, in which the template was copied using Klenow enzyme as described elsewhere,[12] to give a 'pulse' of radioactive label adjacent to the primer. This reaction mixture was divided into two, and half was cleaved with SalPI (or PstI) prior to analysis in parallel with the dideoxynucleotide sequencing reactions. This located the phosphodiester bond cleaved in the cDNA (i.e. non-template DNA) strand. The remaining half was cleaved with SalPI (or PstI) and simultaneously incubated with T4 DNA polymerase and all four deoxyribonucleoside triphosphates. This located the phosphodiester bond cleaved in the template DNA strand. The principle of this method is discussed fully elsewhere.[19]

RESULTS

SalPI purification. After heparin-Biogel and DEAE-cellulose purification the protein concentration of the SalPI preparation was very low: when 1 ml samples (containing at least 6,000 units of SalPI) were concentrated and loaded on SDS-polyacrylamide gels three major, and many less prominent, bands could be seen (data not shown), none of which was present in amounts exceeding 2 μg (judged by the intensity of staining relative to standards). It therefore appears that SalPI is present at a very low protein concentration in S. albus P cells, but has a high activity (i.e. at least 10^3 units per microgram of the enzyme protein). SalPI prepared in this way lost most of its activity after two months at -20°C, regardless of the presence or absence of bovine serum albumen (1 mg/ml). DNA cleaved by such a preparation could be religated by T4 ligase (data not shown) or subjected to effective DNA sequence analysis (see below) indicating that the

SalPI preparation was essentially free of other deoxyribonucleases.

In contrast, phosphocellulose and hydroxylapatite chromatography yielded a preparation which showed no loss of activity after 8 months of storage at -20°C. There was again no evidence of non-specific deoxyribonuclease activity as judged by loss of band sharpness when 0.5 µg pBR322 derivative was analysed by agarose gel electrophoresis after overnight incubation with 30 units of enzyme prepared in this way, and pBR322 DNA cleaved by this SalPI preparation could be ligated to regenerate SalPI target sites (Fig. 1).

SalPI and PstI generate identical tetranucleotide 3' single stranded ends on cleavage of DNA. Sequencing gels were obtained using ØX174 HhaI fragment 4 as primer with (+) strand DNA as template. Fig. 2 shows the regions of these gels that also contained the bands generated by SalPI and PstI cleavage of radioactive DNA made with the same primer-template

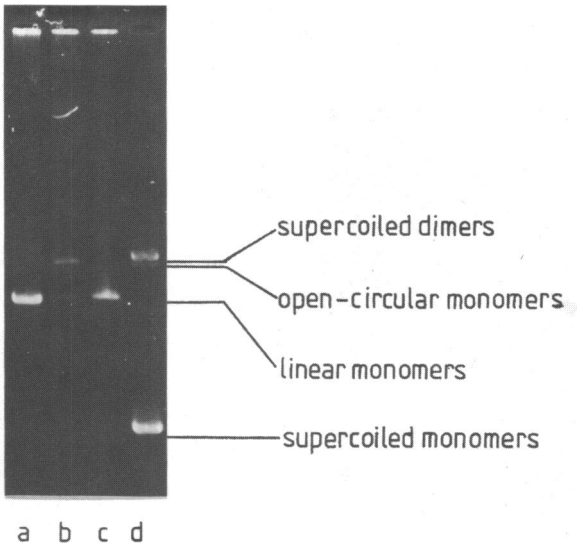

Fig. 1. 1% agarose gel showing re-ligation of pBR322 after cleavage with SalPI. Plasmid pBR322 was cleaved at its single SalPI/PstI target site to give linear monomers (a). These were nearly all converted to open circular monomers on treatment with T4 DNA ligase (b). Further incubation of the ligated DNA with SalPI regenerated linear monomers (c). Track (d) shows untreated pBR 322.

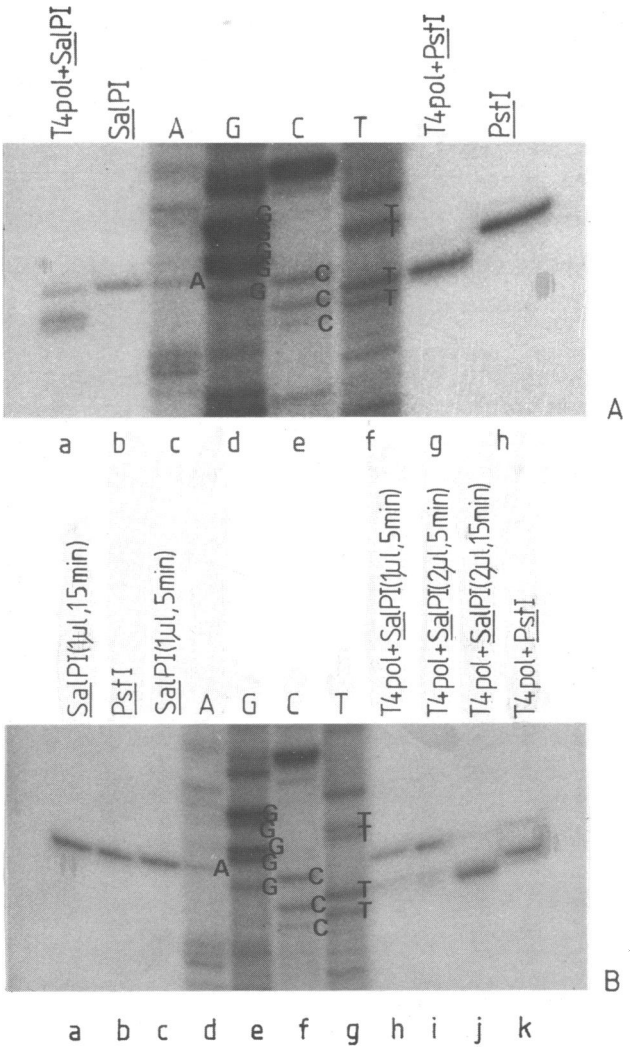

Fig. 2. Determination of mode of cleavage of SalPI on ØX174 RFI DNA. Primer, HhaI fragment 4; template, (+) strand. Note that T4 DNA polymerase (T4 pol) treatment increased the mobility of part, but not all, of the fragments after SalPI or PstI cleavage (A, tracks a and g; B, tracks, h, i, j and k), and that the extent of the conversion was greater after longer incubation with SalPI (B, track j). Also note that this longer incubation did not cause loss of the characteristic SalPI cleavage product (B, track a).

combination. Both enzymes cleaved at the sequence

5'-C-T-G-C-A-G-3'

in the cDNA strand (compare tracks b and h with tracks c, d, e and f for Fig. 2a, and tracks a, b and c with tracks d, e, f and g for Fig. 2b).

After treatment with DNA polymerase and all four deoxyribonucleoside triphosphates, bands were obtained corresponding to the removal of four, three, or no nucleotides from the labelled fragments (Fig. 2a, tracks a and g; Fig. 2b, tracks h, i, j and k). The removal of four nucleotides indicated that SalPI cleaved the double stranded sequence at the same site as PstI:

5'-C-T-G-C-A↓-G-3'

3'-G-↑A-C-G-T-C-5'

The same results were obtained when the primer was HhaI fragment 15 and the template was (-) strand DNA (data not shown).

The occurrence of the bands in which T4 DNA polymerase had removed no nucleotide was probably related to the low activities of both the restriction enzymes. Incomplete digestion might have resulted in the occurrence of an intermediate product with a nick in one strand. Such a product would not be a substrate for T4 DNA polymerase, and if the nick were in the cDNA strand would give rise to a radioactive product of the observed mobility under the denaturing conditions used during electrophoresis. The observation (Fig. 2b, track j) that longer incubation with SalPI and T4 DNA polymerase resulted in the conversion of more of this band to faster migrating forms was consistent with this interpretation.

The "anomalous" band in which T4 DNA polymerase had removed only three nucleotides from the 3' extensions (Fig. 2a, tracks a and g; Fig. 2b, tracks h, i and k) may have been caused by partial specificity and/or low activity of the T4 DNA polymerase. This hypothesis was supported by the observation (Fig. 2b, track j) that a 15 min incubation with SalPI and T4 DNA polymerase, in contrast to the 5 min incubations in tracks h and i, gave almost entirely the form in which four nucleotides had been removed.

Fig. 2b (tracks a and c) also shows that the mobility of the radio-

active fragments used for locating the SalPI cleavage site was unchanged by prolonging the incubation time (in the absence of T4 DNA polymerase). This confirmed that there was little exonuclease activity in the heparin-Biogel purified SalPI preparation used in this experiment.

PstI does not cleave DNA modified in vivo by Streptomyces albus P, and SalPI does not cleave Providencia stuartii chromosomal DNA. It has been shown[3] that DNA extracted from phage R4G2 after propagation on S. albus P is largely resistant to cleavage at the single SalPI target site, which is located 12.75 kb from one end. An experiment was carried out to determine the level of protection of such DNA against PstI cleavage. The experiment (Fig. 3) contained two internal controls: in one, EcoRI, which cleaves R4G2 DNA at a single site near its other end, was also added to the reaction mixtures to allow comparison of the molar ratios of the two end fragments, since otherwise it would have been difficult to exclude the possibility that low apparent levels of SalPI or PstI cleavage were actually due to annealing of the cohesive ends of the phage DNA; and in the other control, DNA of plasmid pAT153 was added to the reaction mixtures to confirm that the reactions had gone to completion. Microdensitometry showed that a six-fold molar excess of the EcoRI 4.5 kb fragment over the SalPI or PstI 12.75 kb fragment was found with S. albus P-modified DNA, but equimolar amounts were obtained with both enzymes when unmodified R4G2 DNA was used. (We had previously observed that 10 - 20% of DNA prepared from R4G2 grown on S. albus P was apparently unmodified.)[3] Complete cleavage of pAT153 was obtained with both enzymes. It therefore appeared that SalPI-specific modification protected the site equally well against both enzymes.

We do not know of a Providencia phage subject to restriction and modification by the PstI system, which would have permitted a reciprocal experiment to be done. However, total DNA of P. stuartii 164 and of S. albus P were uncleaved by either PstI or SalPI, although EcoRI and SalGI cleaved them readily (Fig. 4). This was not due to the presence of an inhibitor in the DNA solutions, as λ DNA was completely cleaved by both PstI and SalPI in their presence (Fig. 4).

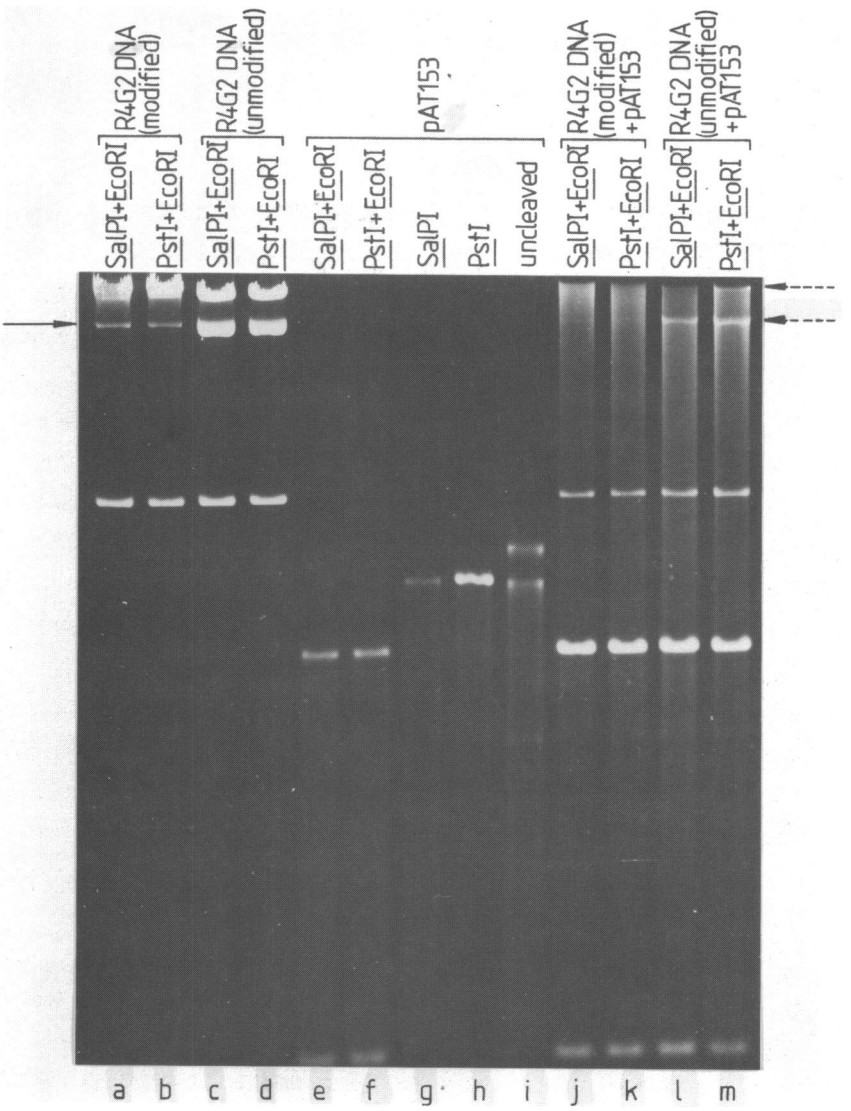

Fig. 3. Protection of phage R4G2 DNA against SalPI and PstI cleavage by pregrowth of the phage on S. albus P. Note that the SalPI/PstI fragment B (unbroken arrow) was present in much reduced quantities when modified R4G2 DNA (i. e. from phage last grown on S. albus P) was used (tracks a and b), although controls with pAT153 showed that digestion had gone to completion (tracks e to m). (A contaminating endonucleolytic activity in the pAT153 preparation caused selective loss of the higher molecular weight bands (dotted arrows) in the mixed digests.) For further explanation, see the text.

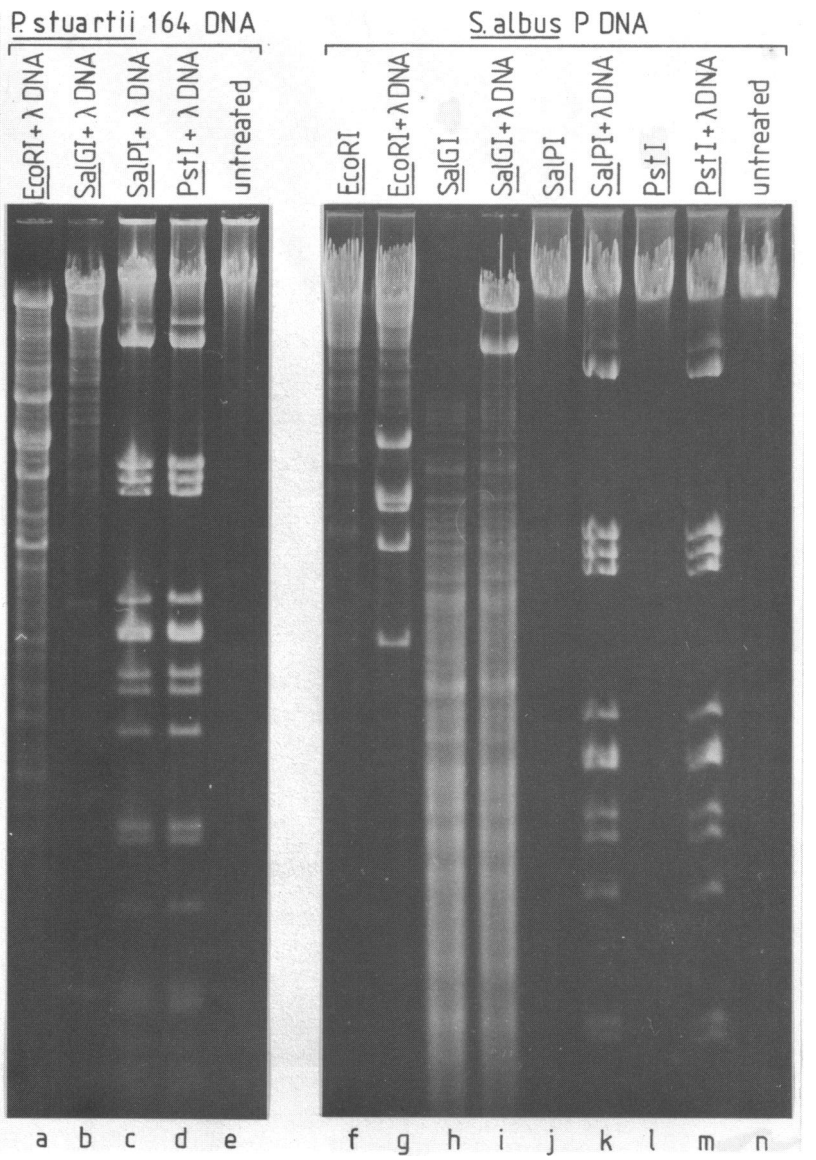

Fig. 4. Resistance of total DNA of Providencia stuartii 164 and Streptomyces albus P to cleavage by SalPI and PstI. SalPI and PstI did not cleave P. stuartii DNA (tracks c, d) or S. albus P DNA (tracks, j, k, l, m) (compare with the untreated DNA in tracks e, n) although cleavage was obtained with EcoRI and SalGI (tracks f, g, h, i). The presence of the P. stuartii DNA or S. albus P DNA did not inhibit cleavage of λ DNA by SalPI (tracks c, k) or PstI (tracks d, m).

DISCUSSION

SalPI and PstI share some properties as proteins and as enzymes; they elute from phosphocellulose and hydroxylapatite at similar positions in salt gradients, and they recognise the same hexanucleotide DNA sequence and cleave the same phosphodiester band within that sequence. There is also some evidence from the sequencing gels (Fig. 2) that it is possible for both enzymes to cleave one strand only of the double stranded target site, indicating that cleavage of the two strands may be independent as for EcoRI[20] rather than co-operative and essentially simultaneous as reported for SalGI.[20] In addition, the associated modification activities present in each producing organism protect DNA from cleavage by the endonuclease from the other (Figs. 3 and 4). These observations raise the interesting possibility of a close evolutionary relationship between the determinants for the two enzymes, such as has been suggested for the determinants of certain drug resistances from Gram-negative bacteria (where they are specified by plasmid or transposon-borne genes) and their counterparts in streptomycetes.[20] This idea might be best tested by cloning the restriction-modification determinants, which should be facilitated in the case of SalPI by the recent establishment of DNA cloning systems in Streptomyces using either plasmid[22, 23] or bacteriophage[24] vectors.

SalPI provides an attractive alternative to PstI for analysis and cloning of DNA. In addition to the ease of obtaining high activities of exonuclease-free enzyme which is relatively stable, the producing organism is a Streptomyces species, and thus a member of one of the least pathogenic groups of bacteria, whereas Providencia stuartii 164 (the source of PstI)[4] was isolated from a clinical source. Spore inocula of S. albus P are easily prepared and can be stably stored in 20% glycerol at -20°C. After growth, the mycelium can conveniently be harvested by filtration and stored at -20°C without apparent reduction in SalPI yield.

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