A DNA fragment from *Streptomyces fradiae* increases the production of a metalloprotease in *Streptomyces lividans*

S MYTHILI and K DH ARM AUNGAM*
Department of Genetic Engineering, School of Biotechnology, Madurai Kamaraj University, Madurai 625021, India

MS received 4 January 1996; revised 1 August 1997

Abstract. *Streptomyces fradiae* produces several extracellular proteases and many of these are inducible. An 88 kb DNA fragment of *Streptomyces fradiae* cloned on pIJ699 caused increased protease activity in *Streptomyces lividans*. Clones carrying this recombinant plasmid showed a significant delay in sporulation. A protein of 18 kDa was purified from the extracellular proteins secreted by the host carrying the recombinant plasmid. Further characterization showed that this protease is a metalloprotease.

Keywords. *Streptomyces*; protease; sporulation.

1. Introduction

*Streptomyces* are Gram-positive, mycelial organisms and they produce several extracellular hydrolases. *Streptomyces* proteases came into focus as a byproduct of antibiotic fermentation (Nomoto and Narahashi 1959; Ginther 1979; Pokorny *et al* 1979; Al-Nuri *et al* 1984; Gibb and Strohl 1988). Protease production seems to be interlinked with the complex regulation of secondary metabolite biosynthesis and sporulation. Genes encoding some of the proteases of *Streptomyces* have been cloned and their nucleotide sequence determined (Henderson *et al* 1987; Chang *et al* 1990; Lampel *et al* 1992; Lichenstein *et al* 1992; Butler *et al* 1993, 1994; Kitadokoro *et al* 1993; Kim and Lee 1995; Taguchi *et al* 1995). *S. fradiae* produces at least five proteinases and these include a keratinase and two exopeptidases (Morihara *et al* 1967). In this paper, we report the cloning of a DNA fragment from *S. fradiae* which increased the production of a metalloprotease in *S. lividans*.

2. Materials and methods

*S. fradiae* (National Chemical Laboratory, Pune) *S. lividans* TK64 and TK23 (Hopwood, John Innes Institute, Norwich, UK) were the *Streptomyces* strains used. *Escherichia coli* strains JM109 and HB101 (Miller 1972) were used for cloning. pIJ699 (Kieser and Melton 1988), pIJ702 (Katz *et al* 1983), pUC18 (Norrander *et al* 1983) and pBR322 (Bolivar *et al* 1977) were used as plasmid cloning vectors.

Restriction enzymes, calf intestinal-alkaline phosphatase and T4 DNA ligase were from New England Biolabs (Beverly, Mo, USA) and Boehringer Mannheim (Mannheim, Germany) and used as described by the manufacturer. Mega-Prime * Corresponding author (Fax, 91–452–859105; Email, mkubic@giasmd01.vsnl.net.in).
labelling kit was from Amersham (UK) and fine chemicals were from Sigma (USA). Other chemicals used were of analytical grade. Thiostrepton was a kind gift from S J Lucania, Squibb and Co. (NJ, USA) and this antibiotic was used at a concentration of 50 μg/ml for agar plates, 5 μg/ml for liquid cultures and 200 μg/ml for selecting transformants. Skimmed milk powder was from Sagar (Anand, India).

Basal salt medium (BSM, Morihara et al 1967), R2YE, YEME (Hopwood et al 1985), and milk broth (Lampel et al 1992) M40 (Polsinelli and Beratta 1966) were prepared as described already. Milk broth contained 0.01% CaCl2. Clearing zones were detected on milk agar containing 1% skimmed milk, buffered with sodium phosphate (pH 7.2).

Streptomyces were grown at 30°C in baffled flasks. Spores and mycelia were maintained as 20% glycerol stocks, at –20°C. Total chromosomal DNA preparation, sucrose gradient fractionation, protoplast preparation and transformation were done as described earlier (Kumaravel and Dharmalingam 1994; Hopwood et al 1985). Plasmid DNA was purified through cesium chloride centrifugation (Maniatis et al 1982) and by using a modified alkaline lysis procedure. Genomic library of S. fradiae was prepared by ligating size fractionated Sau3A digest of chromosomal DNA to pIJ699 DNA digested with BglII and DraI (DraI cleavage prevents the stuffer fragment religation leading to vector formation; Kieser and Melton 1988).

The determination of protease activity was already described (Ginther 1979). Polyacrylamide gel (12%) was used for SDS-PAGE electrophoresis (Laemmli 1970; Ausubel et al 1987) and Polyacrylamide gels ranging from 6 to 12% were used in anionic and cationic systems for zymography (Gabriel 1979). The gels were superimposed onto a 0.8% agarose gel containing 1% milk and 50mM Tris.HCl (pH 7.0), 1 mM CaCl2 and 1 mM ZnCl2 to locate the protein bands which have proteolytic activity.

2.1 Purification of protease

TK23 (pMKU1) was grown in milk broth containing thiostrepton (tsr) up to 72 h. The culture supernatant was filtered through 0.4 µm nitrocellulose filters and concentrated by precipitation with two volumes of acetone. The precipitated proteins were resuspended in 50 mM Tris.HCl (pH 7.5) and dialysed against double distilled water at 4°C overnight, with two changes of water. The sample was adjusted to pH 7.5 DEAE-Sephadex equilibrated with the above buffer was used for ion exchange chromatography. Fractions were assayed for azocaseinolytic activity and the protein concentration was determined using the dye-binding method (Bradford 1976).

3. Results

3.1 Extracellular proteases of S. fradiae

Protease activity in S. fradiae NCL was maximum (17.8 U/mg) at 36 h of growth in BSM. In this experiment the inoculum was spores (5 × 10⁷ spores per 100 ml of medium). The specific activity of the protease decreased after 48 h of growth indicating the absence of synthesis of the enzyme during the later stages of growth, even though the extracellular protein level increased during that period (data not shown). However, total protease activity (U/ml) remained high, perhaps due to the stability of the protease
Enhanced production of a metalloprotease in S. lividans protein. In some experiments the initial inoculum was mycelia grown in YEME and in this case the protease activity was lower. In these experiments mycelia showed fragmentation after 48 h and the total extracellular protein production was also quite low (data not shown). However, when mycelia were cultivated in milk broth medium, protease activity was about 40-fold higher at 96h of growth. Repeated experiments confirm the pattern of activity described above and fragmentation of mycelia was also absent. This implies that protease (s) were induced in cultures grown in milk broth.

Results (data not shown) showed that the extracellular protease activity was maximum at 50°C and in subsequent experiments carried out at this temperature, it was observed that the enzyme exhibited a broad pH range (7 to 10.6). The extracellular proteases were inhibited by EDTA and Phenyl methyl sulfonyl fluoride (PMSF) (80% and 60% inhibition, respectively) indicating that the proteases of S. fradiae are predominantly a mixture of metallo and serine protease (s).

3.2 Protease production in S. Hvidans was increased by a DNA fragment cloned from S. fradiae

A genomic library of S. fradiae was constructed in pIJ699, as described in § 2. S. lividans TK64 carrying the recombinants were screened for protease production in milk agar plates since the host S. lividans did not show significant protease activity (figure 1) in the above assay. Two transformants among the 2000 screened showed large clearing zones after incubation for 120h indicating the increased protease production. One of the transformants had undergone DNA re-arrangement including amplification of a segment of chromosomal DNA. The other transformant carried a recombinant plasmid (pMKU1) increased protease production when retransformed into S. lividans TK23.

Figure 1. Plates showing the zones of hydrolysis by S. lividans TK23 (pIJ699) and TK23 (pMKU1) in milk-agar plates containing 50 μg/ml thiostrepton. The plates were incubated at 30°C for 120 h.
Figure 2. Chromosomal DNA digested with restriction enzymes were run on 1% agarose and stained with ethidium bromide (lanes 1–7). Fragments were then transferred to nitrocellulose membrane and Southern hybridization was done using as probe the cloned insert from pMKU1 labelled with [³²P]dCTP (lanes 1’ to 7’). Lane 1, HindIII; lanes 2, 3, 4 and 5, S. fradiae chromosomal DNA cleaved with BamHI, BglII, SalI and PstI, respectively; lane 6, S. lividans TK23 chromosomal DNA digested with BamHI; lane 7, p MKU1, digested with HindIII.

Metalloproteases were shown to have regulatory elements with DNA sequence homology to lysR family of regulators (Damman and Wohlleben 1992). Further, the zinc proteases have a conserved zinc binding domain that was shown to be present in S. lividans TK23 (Damman and Wohlleben 1992). In order to determine whether the cloned S. fradiae fragment has sequences homologous to the chromosomal DNA of S. lividans, we analysed the chromosomal DNA using [³²P]-labelled pMKU1 as probe. Results in figure 2 show that the probe hybridized only to the chromosomal DNA of S. fradiae and not to that of S. lividans TK23.
3.3 Sporulation is affected in the cells carrying pMKU1

*S. lividans* TK64 carrying pMKU1 showed poor sporulation in rich media like milk agar, R2YE and soyabean mannitol agar plates. Excess extracellular protease production is likely to be the reason for the delay in sporulation because of the efficient conversion of extracellular proteins to usable carbon and nitrogen sources. However, the clone showed delay in germination of spores in M40 minimal medium also. When compared to *S. lividans*, *S. fradiae* also showed delay in germination on M40 plates, however, once germinated *S. fradiae* sporulates much faster than *S. lividans* carrying pMKU1. This delay is linked to protease production of the clones, since the variants which sporulated well always showed reduced protease activity.

3.4 Protease production by *S. lividans* TK23 (pMKU1)

Extracellular protease production by *S. lividans* TK23 (pMKU1) was compared with that of *S. fradiae* and *S. lividans* TK23, in BSM and in milk broth using mycelia as inoculum. In BSM, TK23 (pMKU1) showed 5-fold higher protease activity than *S. fradiae* (figure 3). However, the rate of extracellular protein synthesis was two-fold

![Figure 3. Extracellular protease activity and protein production in the strains grown in BSM. Mycelia were used as initial inoculum. Protease activity was assayed in the culture filtrate at pH 7.0, at 50°C as described in § 2. Protein concentration estimated by Lowry’s method (Lowry et al 1951). (**—**), Protease U/ml; (———), protein µg/ml; (*), *S. fradiae*; (□), *S. lividans* TK23; (*), TK23 (pMKU1).]
higher in TK23 (pMKU1) and hence the level of specific activity is not much higher in TK23 (pMKU1). The protease activity was much lower in TK23 (figure 3). Whereas in milk broth, there was a two-fold increase in activity in TK23 (pMKU1) compared to TK23 but the protease activity was lower than that in S. fradiae (figure 4). In TK23 and S. fradiae, protease production was higher when grown in milk broth, but not in TK23 (pMKU1). The fold of increased protease production based on the data given in figures 3, 4 and from other results was 146, 111 and 2 in S. fradiae, S. lividans TK23 and TK23 (pMKU1) respectively, at 72h of growth. Statistical analysis of data from multiple experiments, confirm the above conclusion. These results indicate the altered regulation of protease production in the host carrying the recombinant plasmid since protease production was not significantly induced in milk broth. There was also an enhanced production of extracellular proteins in TK23 (pMKU1).

Thiostrepton was routinely added to the medium, used for growing S. lividans harbouring plasmids. On milk agar plates, TK23 (pIJ699) showed small but distinct clearing zone (figure 1) than S. lividans TK23 (result not shown). Thiostrepton was shown to induce several genes in S. lividans (Murakami et al 1989). Therefore, to rule
out the possibility that the enhanced production in the recombinant clone is due to the antibiotic, protease activity was assayed in cultures [TK23 (pIJ699), TK23 (pMKU1)] grown in milk broth in the presence and absence of thiostrepton. Figure 4 shows that thiostrepton did not induce protease expression in TK23 (pIJ699) and TK23 (pMKU1). Murakami et al (1989) reported the induction of specific intracellular proteins by thiostrepton. TK23 (pIJ699) cells grown in the presence of thiostrepton showed induction of extracellular proteins as well (figure 5). In TK23 (pMKU1) such induced proteins were present intracellularly but not in the extracellular medium (data not shown). These proteins, apparently, could have been degraded by the protease expressed in TK23 (pMKU1).

3.5 Characterization of the protease from TK23 (pMKU1)

The pH optimum for the protease of TK23 (pMKU1) was 7.0, and nearly 70% activity was detectable at pH 9.0. The temperature optimum was 50°C, and the activity was also
Table 1. Effect of inhibitors and metal ions on the protease activity from TK23 (pMKU1).

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Additions</th>
<th>Protease activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nil</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Isopropanol</td>
<td>19.6</td>
</tr>
<tr>
<td>3</td>
<td>PMSF</td>
<td>112.2</td>
</tr>
<tr>
<td>4</td>
<td>EDTA</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>EGTA</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>PHEN</td>
<td>14.7</td>
</tr>
<tr>
<td>7</td>
<td>DTT</td>
<td>17.8</td>
</tr>
<tr>
<td>8</td>
<td>EDTA</td>
<td>37.29</td>
</tr>
<tr>
<td>9</td>
<td>EDTA, Ca(^{2+})</td>
<td>55.50</td>
</tr>
<tr>
<td>10</td>
<td>EDTA, Mg(^{2+})</td>
<td>35.82</td>
</tr>
<tr>
<td>11</td>
<td>EDTA, Zn(^{2+})</td>
<td>74.88</td>
</tr>
<tr>
<td>12</td>
<td>EDTA, Ca(^{2+}), Mg(^{2+})</td>
<td>51.39</td>
</tr>
<tr>
<td>13</td>
<td>EDTA, Ca(^{2+}), Zn(^{2+})</td>
<td>93.24</td>
</tr>
</tbody>
</table>

In experiments 1–6, culture filtrate was used. For experiments 7–12 the culture filtrate dialysed for 12h against double distilled water. Two nM each CaCl\(_2\), MgCl\(_2\) and ZnCl\(_2\) was used in experiments 8–12. The concentrations of inhibitors used were, PMSF (1mM), EDTA (10mM), EGTA (10mM), PHEN (1mM) and DTT (1mM).

stable on storage of the culture broth at room temperature (30 to 35°C) over a period of a month. However, the protease activity was inactivated completely when incubated for 1 h at 70°C.

Studies using inhibitors showed that the above protease was a metalloenzyme. The inhibition was 100% with 10 mM EDTA, 100% with 10 mM phenanthroline (PHEN) 96.7% with 10 mM ethylene glycol tetra acetic acid (EGTA) and 82% with 1 mM dithiothreitol (DTT). One mM phenyl methyl sulphonyl fluoride (PMSF) in 10% isopropanol enhanced activity to 112%. Since 10% isopropanol itself inhibits protease activity to some extent, this enhancement by PMSF was significant. To determine the metal required for the above protease, we added metal ions to EDTA inhibited enzyme preparation, before the assay. Table 1 shows that zinc alone or in combination with calcium could restore the enzyme activity, while magnesium had no effect. Further, the enzyme did not lose activity after extensive dialysis against water nor after acetone precipitation. These results indicate that the metalloprotease likely has bound zinc and calcium.

Activity gels were run as described in § 2. The regular native gels in anionic system did not resolve the clearing bands distinctly and the enzyme did not enter the separating gel. This is probably due to its net positive charge conferred by the bound metal ions and hence the cationic system of Gabriel (1979) was tried. A sharp clearing band corresponding to molecular weight marker trypsinogen was found both in S. fradiae and TK23 (pMKU1). Below this band diffuse bands were observed (data not shown). No clearing bands were observed for TK23 (pIJ699). The purified sample (table 2 showed a single band of 18 kDa on SDS-PAGE both with Coomassie blue and silver staining (figure 6).
Enhanced production of a metalloprotease in S. lividans

Table 2. Purification of the metalloprotease from TK23 (pMKU1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein conc. (µg/ml)</th>
<th>Protease (U/ml)</th>
<th>Protease (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>ND</td>
<td>3.285</td>
<td>---</td>
</tr>
<tr>
<td>Concentrate</td>
<td>271</td>
<td>13.48</td>
<td>49.7</td>
</tr>
<tr>
<td>Dialysate</td>
<td>173</td>
<td>12.12</td>
<td>70.96</td>
</tr>
<tr>
<td>DEAE eluate</td>
<td>2.7</td>
<td>7.28</td>
<td>2696.29</td>
</tr>
</tbody>
</table>

TK23 (pMKU1) was grown in milk broth containing thiostrepton. Protein concentration was determined by Bradford’s dye-binding assay. Protease assayed by azocaseinolysis as given in § 2. Details are described under § 2.

ND, not determined.

Figure 6. Extracellular proteins of TK23 (pMKU1) separated on SDS-PAGE, stained with Coomassie blue. The sample volume equivalent to 12 U of protease activity was loaded per slot. Lane 1, Concentrated sample; lane 2, dialysate; lane 3, DEAE eluate and lane 4, molecular weight markers.

4. Discussion

*S. fradiae* constitutively secretes several extracellular proteases during the vegetative phase, whereas in other *Streptomyces*, protease production is generally associated with
sporulation (Pokorny et al. 1979; Ginther 1979; James et al. 1991). This correlation, however could be coincidental in many cases because one could isolate protease hyperproducing, non-sporulating mutants (Gibb et al. 1987, 1989). Moreover, for the bacteria to survive on organic debris proteases must be made during vegetative phase of growth. Apart from proteases, Streptomyces secrete a large number of hydrolytic enzymes and many of them are inducible by the substrate (Peczynska-Czoch and Mordarski 1988). The high level protease production by \textit{S. fradiae}, observed in milk medium could be due to the induction of proteases, however the number of different proteases induced was not determined.

The enzyme produced by TK23 (pMKU1) is unique in that it is activated by PM SF. Similarly DTT inhibits the activity as in the case of the metalloprotease of \textit{B. subtilis} (Rufo et al. 1990). The protease activity from TK23 (pMKU1) shows a pH optimum of 7.0 while the native enzymes of \textit{S. lividans} are not fully active at neutral pH (Lichenstein et al. 1992; Aphale and Strohl 1993). The zinc metalloprotease (leucine aminopeptidase) of \textit{S. lividans} 1326 is known to be stabilized, but not activated by calcium (Aphale and Strohl 1993). The zinc metalloprotease of \textit{S. lividans} TK23 (pMKU1) is activated by calcium. In this regard it rather resembles the aminopeptidases of \textit{S. peptidofaciens}, \textit{S. griseus} and \textit{S. rimosus} which require calcium for activity (Spungin and Blumberg 1989; Renko et al. 1981; Vitale et al. 1986).

Germination of spores is retarded in clones carrying pMKU1. This is analogous to the effect of the gene \textit{saf} from \textit{S. griseus} which not only delays sporulation but also pigment production when cloned in a heterologous host (Daza et al. 1990). Further, \textit{saf} was shown to increase production of several extracellular enzymes, including protease. \textit{Saf} hybridizes with the DNA of several \textit{Streptomyces} species including \textit{S. fradiae}. Unlike \textit{saf}, the insert in pMKU1 does not hybridize with \textit{S. lividans} DNA. Further, pMKU1 does not affect pigmentation in \textit{S. lividans}.

The extracellular protein profile of TK23 (pMKU1) (SDS-PAGE) showed that the number of protein bands are limited, unlike in \textit{S. lividans} TK23 and TK23 (pIJ699). This was in contrast to the high concentration observed by in \textit{vitro} protein assay of the TK23 (pMKU1) culture filtrate. It is most likely that the protease degrades the extracellular proteins secreted by the host. This could also explain the ease of purification of pMKU1 encoded protease, an 18 kDa protein. Based on these results the 8.8 kb \textit{S. fradia} DNA insert in pMKU1 encodes a new protease protein of 18 kDa in \textit{S. lividans}.

References


Enhanced production of a metalloprotease in \textit{S. lividans}


Chang P C, Kuo T C, Tsugita A and Lee Y H N 1990 Extracellular metalloprotease gene of \textit{Streptomyces cacaoi}, structure, nucleotide sequence and characterization of the cloned product; \textit{Gene} 88 87–95

Damman T and Wohleben W 1992 A metalloprotease gene from \textit{Streptomyces coelicolor} ‘ Muller’ and its transcriptional activator, a member of the \textit{LysR} family; \textit{ Mol. Microbiol.} 6 2267–2278


Gabriel O 1979 Analytical disc gel electrophoresis; \textit{ Methods Enzymol.} 22 565–578


Kim I S and Lee K J 1995 Physiological roles of leupeptin and extracellular proteases in mycelium development of \textit{Streptomyces exfoliatus} SMF13; \textit{Microbiology} 141 1017–1025


Kumaravel S and Dharmalingam K 1994 structural organization, amplification, deletion and rearrangements of DNA sequences associated with an unstable region of the chromosome of \textit{Streptomyces coelicolor} A3 (2); \textit{Indian J. Biochem. Biophys.} 31 280–287

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4; \textit{Nature (London)} 227 680–685


Miller J 1972 \textit{Experiments in molecular genetics} (New York: Cold Spring Harbor Laboratory)

Morihara K, Oka T and Tsuzuki H 1967 Multiple proteolytic enzymes of \textit{Streptomyces fradiae}. Production, isolation and preliminary characterization; \textit{Biochim. Biophys. Acta} 139 382–397


Nornoto M and Narahashi Y 1959 A proteolytic enzyme of \textit{Streptomyces griseus}; \textit{J. Biochem.} 46 653–667

Norlander J, Kemp T and Messing J 1983 Construction of improved M13 vectors by oligonucleotide-directed mutagenesis; \textit{Gene} 26 101–106
Polsinelli M and Beretta M 1966 Genetic recombination in crosses between Streptomyces aureofaciens and Streptomyces rimosus; J. Bacteriol. 91 63–68
Spungin A and Blumberg S 1989 Streptomyces griseus aminopeptidase is a calcium-activated zinc metalloprotein. Purification and properties of the enzyme; Eur. J. Biochem. 183 471–477

Corresponding editor: M S SHAILA