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A linear plasmid temperature-sensitive for replication in *Streptomyces hygroscopicus* 10-22

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Abstract

Streptomyces hygroscopicus 10-22 harbors a conjugative, autonomously replicating linear plasmid pHZ6 of ca. 70 kb, which shows no obvious homology with chromosomal DNA and is temperature-sensitive for replication, being stable in the host at 28°C but easily lost at 37°C. On a lawn of the wild-type *S. hygroscopicus* 10-22 cured of pHZ6, pHZ6 elicit pocks. Temperature sensitivity seemed to be a unique property for pHZ6 among six linear plasmids tested, including the well-known linear plasmids SLP2 in *Streptomyces lividans* 1326 and SCP1 in *Streptomyces coelicolor* A3(2). The distinct identity of pHZ6 from previously identified pHZ1–pHZ5 was demonstrated by the profile of relevant plasmids in six well-defined strains originated from *S. hygroscopicus* 10-22.

Keywords: Linear plasmid; Temperature-sensitive; *Streptomyces*

1. Introduction

Most of the extra-chromosomal DNA elements isolated in *Streptomyces* were found to exist in covalently closed circular (CCC) forms before large linear plasmids could be detected by pulsed field gel electrophoresis (PFGE) [1]. *Streptomyces rochei* was the first *Streptomyces* strain in which two linear plasmids, pSLA1 and pSLA2, were detected and characterized [2]. Small linear plasmids were also found in *S. azureus* [3], *S. rimosus* [4] and *S. clavuligerus* [5]. Large linear plasmids include SCP1 (350 kb) in *S. coelicolor* A3(2) [1], pKSL (520 kb) in *S. lasaliensis* [1], pSA1 (100 kb) and pSA2 (250 kb) in *S. avermitilis* [6] and those in β-lactam antibiotic-producing *Streptomyces* [7]. Some of them were first identified genetically [8], before being physically detected by PFGE. The *Streptomyces* linear plasmids so far documented share common features with bacteriophages, adenovirus and linear plasmids from yeast in containing terminal inverted repeat sequences, with terminal proteins attached at both ends [9].

Among the *Streptomyces* plasmids studied, including both circular and linear molecules, only pSG5 from *S. ghanaensis* was identified as a naturally temperature-sensitive entity [10], which could not replicate above 34°C and thus resulted in gradual loss in successive culture. This feature has made it possible to develop a new vector system used widely in gene disruption and mutational cloning experiments in *Streptomyces* [10]. pMT660, a temperature-sensitive mutant plasmid of pIJ702, was also widely used in *Streptomyces* in this respect [11]. To our knowledge, however, no example of a temperature-sensitive linear plasmid in *Streptomyces* and closely related actinomycetes has been reported.

Here, we describe the identification and characterization of a temperature-sensitive linear plasmid in *S. hygroscopicus* 10-22, a strain known to produce at least four antifungal antibiotic compounds and to contain five plasmids, four of which were characterized as CCC molecules [12].

2. Materials and methods

2.1. Bacterial strains and plasmids

Streptomyces strains used are *S. hygroscopicus* 10-22 (pHZ6$^+$), N-103 (pHZ6$^+$), Q303 (pHZ6$^+$), Q105 (pHZ6$^-$) and Q1513 (pHZ6$^-$) [12]. Linear plasmids pHZ226 (ca. 140 kb, unpublished) from *S. hygroscopicus*

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5008, pHZ227 (ca. 130 kb, unpublished) and pHZ228 (ca. 30 kb, unpublished) from *Streptomyces* sp. FR-008, SLP2 (50 kb) from *S. lividans* [13] and SCP1 (350 kb) from *S. coelicolor* [14] were used for comparative studies as linear molecules. PXH1 is a derivative of *S. hygroscopicus* 10-22 cured of pHZ6, resulting from the present study.

2.2. Conjugal transfer

Spores of *S. hygroscopicus* 10-22 and its derivatives were mixed and spread on HAUCM plates [12] and the cultures were grown at 28°C for 24 h, during which pocks could become visible.

2.3. Culture conditions and PFGE

YEME medium [15] containing 34% (w/v) sucrose was used for the preparation of *S. lividans* and *S. coelicolor* A3(2) and YEME medium containing 10.3% (w/v) sucrose [12] was used for the cultivation of *S. hygroscopicus* 10-22, *S. hygroscopicus* 5008 and *Streptomyces* sp. FR-008. *S. hygroscopicus* 10-22 and its derivatives were maintained on HAUCM plates [12] at 28°C or 37°C.

We used the CHEF-DRIII system supplied by Bio-Rad for PFGE analysis. Electrophoresis was carried out at 14°C with different switching intervals in 0.5×TBE (44.5mM Tris, 44.5 mM boric acid, 1 mM EDTA) buffer with 0.8–1% agarose gels. For preparation of non-proteolytically treated samples, proteinase K was omitted and plugs were incubated in 2% SDS, 0.5 M EDTA (pH 8.0) as described by Lin et al. [16].

2.4. Southern transfer and hybridization

DNA from PFGE was nicked under 254 nm UV for 30 s, and depurinized in 0.25 N HCl for 15 min, washed with distilled water three times and then denatured in 0.4 N NaOH, 1.5 M NaCl for 30 min, followed by neutralization in 0.5 M Tris–HCl, 1.5 M NaCl (pH 7.0) for 15 min and then transferred to nylon membranes using a vacuum transfer system (Bio-Rad). The DNA probe was recovered by electroelution, labeled with [^{32}P]α-dCTP and detected with a phosphoimager (Fujifilm).

3. Results and discussion

3.1. Detection of a temperature-sensitive and autonomously replicating linear plasmid, pHZ6, in S. hygroscopicus 10-22

PFGE of *S. hygroscopicus* 10-22 mycelium plugs embedded in low-melting agarose under conditions suitable for the separation of linear molecules revealed a clear band, ca. 70 kb in size (Fig. 1A, lane 2). When plugs were treated with either proteinase K or SDS, the DNA band could only be visualized in the proteinase K-treated sample, but not in the sample treated with SDS, suggesting that the observed band is likely to be a linear plasmid with covalently bound proteins at the ends. This plasmid was tentatively named pHZ6.

Spores of *S. hygroscopicus* 10-22 were inoculated into YEME and incubated in an orbital shaker at 28°C or 37°C. The mycelium was then harvested, used to prepare plugs, and subjected to PFGE. pHZ6 could only be detected with mycelium grown at 28°C but not at 37°C (Fig. 1B, lane 5), implying that the replication of pHZ6 is temperature-sensitive.

To confirm the temperature sensitivity of pHZ6, spores of *S. hygroscopicus* 10-22 were streaked onto HAUCM medium and incubated at 37°C. Single colonies were isolated and streaked onto the same medium for five rounds before 10 single colonies were picked and inoculated into YEME liquid medium and incubated at 28°C. The resulting mycelia were collected and used to make plugs for the detection of pHZ6. None of the 10 samples contained the linear plasmid pHZ6. One of the 10 single colonies was selected and designated PXH1. The *Ase*I digestion profile of PXH1 was identical to that of 10-22, showing that PXH1 was derived from 10-22 (data not shown).

When wild-type strain 10-22 was grown on a lawn of any of the 10 single isolates (PXH1–10), obvious pocks, typical of many conjugative plasmids [17], could be clearly seen, suggesting that the linear plasmid pHZ6 is also conjugative. However, an effort to detect pHZ6 by PFGE after reintroduction into pHX1–10 (as well as introduction into a *S. lividans* 66 derivative, ZX1) by conjugation consistently failed, because other circular plasmids pHZ1–4 (perhaps the putative integrative pHZ5 as well) [12] orig-

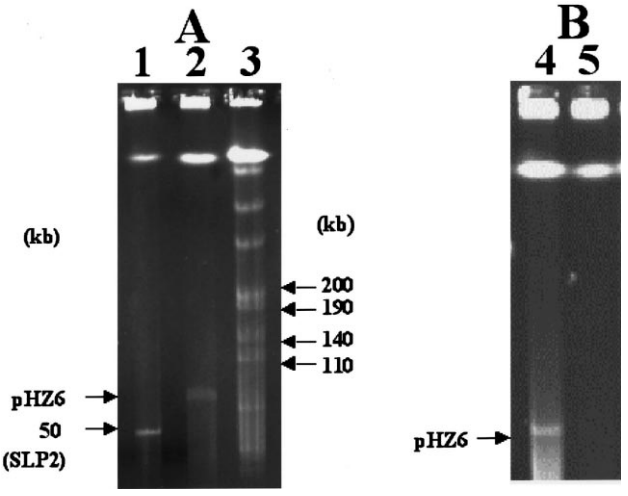

Fig. 1. PFGE analysis of the genome of *S. hygroscopicus* 10-22 carrying a linear plasmid. A: 50 kb of SLP2 of *S. lividans* 1326 (1) and *Ase*I-digested *S. coelicolor* M145 (3) [22] were run in parallel as size standards. pHZ6 from *S. hygroscopicus* 10-22 (2) is ca. 70 kb in size. B: pHZ6 was only detectable with a sample incubated at 28°C (4) but not at 37°C (5). The run time was 14 h at 6 V cm^{-1} with a 10–40 s switch time at an included angle of 120°.

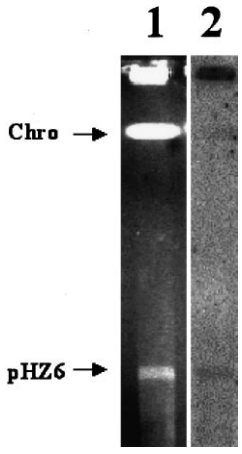

Fig. 2. No DNA homologous with pHZ6 is present on the chromosome of *S. hygroscopicus* 10-22. Lane 2 is the Southern blot of the PFGE of *S. hygroscopicus* 10-22 (1) hybridized with radioactively labeled pHZ6. No hybridizing signal corresponding to the position of chromosomal DNA can be seen.

inating from wild-type strain 10-22, which was used as the donor for conjugation, dominated the transconjugants. The failure of detection could be well explained by the fact that pHZ6 has no known selection marker(s) other than pock formation and dependence of its detection by PFGE technique whose large-scale screening is not practically feasible and thus could be a major limiting factor.

Linear or circular plasmids may integrate into [18–20] or loop out from the bacterial chromosome [18] and might also share homology, e.g. to terminal sequences, with the chromosome [21]. To examine the potential origin of pHZ6, the genomic DNA of *S. hygroscopicus* 10-22 was probed with ^{32}P-labelled pHZ6 recovered from PFGE (Fig. 2). The probe hybridized only to itself, indicating that no copy of pHZ6 or significant homologous sequence is present in the chromosome. Thus, pHZ6 is an autonomously replicating linear plasmid in strain 10-22.

3.2. pHZ6 is independent of any of the indigenous plasmids detected in previous studies

The independence of pHZ6 from pHZ1–5 was demonstrated by the status of plasmids in 10-22 and some of its well-defined mutant strains cured of individual plasmids. Four circular plasmids (pHZ1, 60 kb; pHZ2, 4.7 kb; pHZ3, 4.1 kb; and pHZ4, 3.3 kb) were detected in *S. hygroscopicus* 10-22, and they were all demonstrated to be CCC molecules by two-dimensional gel electrophoresis [12]. In principle, pHZ6 would be independent of pHZ1–4, because the linear molecules would be retarded in the gel slot and would thus be undetectable by conventional gel electrophoresis. The fifth plasmid, pHZ5, whose existence was originally implied by pock formation of the 10-22-derived strain Q105 on a lawn of another 10-22-derived strain Q303, was also ruled out as equivalent to pHZ6 because pHZ6 is detectable by PFGE in Q303 but not in Q105, a situation opposite to the correlation with pock-forming activity elicited by pHZ5 (Table 1).

Table 1 summarizes the plasmid profiles of some 10-22-derived strains, as elucidated by physical isolation and pock formation. pHZ1, pHZ5 and pHZ6 all seemed to be conjugative because they all formed pocks on a lawn of the strains cured of the relevant plasmids and three clearly distinguishable types of pocks could be observed. The three smallest plasmids, pHZ2–4, are physically detectable as CCC molecules in all of the strains listed in Table 1 and were thus not included.

The overall plasmid profiles of pHZ1, pHZ5 and pHZ6 in 10-22, PXH1, N103, Q303, Q105 and Q1513 were clearly distinct from each other. It could thus be concluded that pHZ1, pHZ5 and pHZ6 are all independent plasmids in strain 10-22.

Table 1
The plasmid profile and pock resistance of 10-22 and some of its derivatives

	10-22	PXH1	N103	Q303	Q105	Q1513
pHZ1	+	+	+	−	−	−
pHZ5	+	+	−	−	+	−
pHZ6 (L)	+	−	+	+	−	−

+ (−): relevant plasmid present (absent) and pock-resistant (-sensitive) to the plasmid. L: detected as linear plasmid.

3.3. Temperature sensitivity is a unique property of pHZ6 among six linear plasmids tested

Is temperature sensitivity a unique property of pHZ6? We addressed this question by selecting six linear plasmids

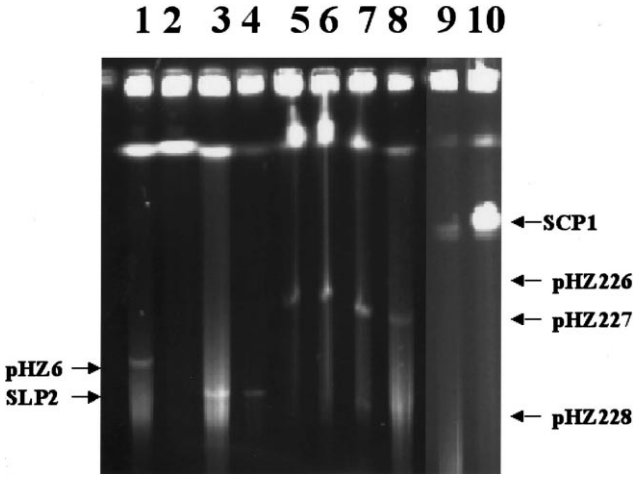

Fig. 3. Detection of pHZ6 of *S. hygroscopicus* 10-22 at 28°C (1) and 37°C (2), 50 kb of SLP2 of *S. lividans* 1326 at 28°C (3) and 37°C (4), 140 kb of pHZ226 of *S. hygroscopicus* 5008 at 28°C (5) and 37°C (6), 130 kb of pHZ227 and 30 kb of pHZ228 of *Streptomyces* sp. FR-008 at 28°C (7) and 37°C (8), and 350 kb of SCP1 of *S. coelicolor* A3(2) at 28°C (9) and 37°C (10) by PFGE. Only pHZ6 was seen to be lost after temperature shift to 37°C. The run time was 17 h at 6 V cm^{-1} with a 10–50 s switch time at an included angle of 120°.

for parallel experiments. Spores of *S. lividans* 1326 carrying SLP2 (50 kb) [21], *S. coelicolor* A3(2) carrying SCP1 (350 kb) [1], *S. hygroscopicus* 5008 carrying pHZ226 (140 kb) (unpublished data) and *Streptomyces* sp. FR-008 carrying pHZ227 (130 kb) and pHZ228 (30 kb) (unpublished data), together with 10-22 carrying pHZ6, were inoculated into YEME, and incubated at 28°C and 37°C, before being subjected to PFGE (Fig. 3). None of the linear plasmids tested, except pHZ6, was found to exist only when cells were grown at 28°C but lost at 37°C, indicating that pHZ6 was unique in this respect. Interestingly, the copy number of SCP1 from *S. coelicolor* A3(2) seemed to be at least 10 times higher when cells were grown at 37°C than grown at 28°C (Fig. 3, two independent repeat experiments gave the same results).

Acknowledgements

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