African Journal of Biotechnology Vol. 7 (17), pp. 3155-3161, 3 September, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Protoplasting impact on polyketide activity and characterization of the interspecific fusants from *Streptomyces* spp.

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Accepted 29 October, 2007

The influence of protoplasting and protoplast regeneration on antibiotic activity, transfer of biosynthesis encoding genes in local *Streptomyces* spp. CN207 was studied. The frequency of regenerated protoplasts in the lag phase was $1.7x10^3$ CFU/ml, in the beginning of the exponential phase $0.4x10^2$ CFU/ml, in the exponential growth phase $2.5x10^3$ CFU/ml, and $1.0x10^5$ CFU/ml in stationary phase. The protoplast formation and regeneration technique resulted in a new isolate strain of *Streptomyces* spp.PR01 that produced approximately 5 fold more *Streptomyces* spp. CN207 antibiotic. The protoplast fusion resulted in increased isolation of variants with higher antibiotic activity. Recombinant *Streptomyces coelicolor* PF04 was increased 10 times more than the wild strain. The processes also affected on the strain resistance to some antibiotics but had no effect on the components of the antibiotic. The characteristics of this recombinant product were similar considerably to *Streptomyces* spp. CN207 product. Our data, in principal, indicate the possibility of transferring antibiotics cluster genes by fusion and provide a starting point for genetic and biochemical investigations of CN207 biosynthesis.

Key words: *Streptomyces*, polyketide antibiotic, gene transfer, protoplast regeneration, fusion.

INTRODUCTION

The study of genetics of *Streptomyces* is important not only because this genus produces many antibiotics but also because its differentiation and its regulation of secondary metabolism are of basic interest. *Streptomyces* species are mycelial, gram-positive bacteria that are readily isolated from soil and produce a diverse range of antibiotics. Gene transfer between bacteria has been widely documented for traits under environmental selection for example, antibiotic resistance (Sundin et al., 1995; Archer et al., 1994; Maynard et al., 1991).

The protoplast formation and regeneration are important processes, and they are a major step following genetic manipulations such as fusion and DNA-mediated transformation, which can improve antibiotic production (Adrio and Demain, 2006; Liras and Rodriguez-Garcia, 2000).

Protoplast fusion technique has been used widely among interspecies or intergenetic microorganisms (Verma et al., 1992; Kirimura et al., 1989). As method for improving the capabilities of microbes, the fusion technique has aroused a good deal of interest for breeding interspecific as well as intergenetic hybrids. Sermonti and Spada-Sermonti (1955) were the first to report about recombination studies on *Streptomyces* using *Streptomyce coelicolor*. The protoplast fusion can be used to regenerate strains with increased antibiotic activity (Hui et al., 1997; Illing et al., 1989). Our collection isolates were identified belonging to genus of *Streptomyces* (Kitouni et al., 2004; Rintala et al., 2001). *Streptomyces* spp. CN207

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produces a natural wide spectrum antimicrobial activity which belongs to family of polyketide antibiotics (Esmanhoto and Kilikian, 2004).

This paper explains the relation between productivity and optimization of conditions for protoplast formation and regeneration of *Streptomyces* spp. CN207. Higher productivity was investigated and characterized in recombinants using chemical and molecular methods.

EXPERIMENTS

Strains and media

Local *Streptomyces* spp. CN207 was used as a donor and *S. coelicolor* M145 was used as a recipient host for protoplast fusion (Kieser et al., 2000). *Streptomyces lividans* 1326 (*Scb A*$^+$) was used for detecting *Scb*A gene (Butler et al., 2003). Nutrient agar was used to prepare spore suspensions, YEME medium was used for the growth culture (Kieser et al., 2000) and protoplasts regeneration was accomplished on R2YE plates (Yang and Lei, 2001). Cultures of *S. coelicolor* M145 were grown essentially as described by Strauch et al. (1991). For antimicrobial CN207 production, *Streptomyces* spp. CN207 was grown on natural medium bran-barley media and initial inoculums 1x10^6 spore/ml at 28ºC and 120 rpm shaking for five days. *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29219 sensitive strains were used for antimicrobial detection in this work. These strains were grown on Mueller Hinton medium (MH).

Antimicrobial susceptibility testing

All *Streptomyces* strains were subjected to susceptibility, testing to the antibiotics in Table 2 by discs diffusion (Bio-Rad disc).

Protoplast preparation and regeneration

Streptomyces spp. CN207 and *S. coelicolor* M145 protoplast preparation and regeneration were based on published methodologies (Thompson et al., 1982; Hopwood and Wright, 1978). The protoplast formation was monitored microscopically. Regeneration colonies of *Streptomyces* spp. CN207 were examined for over production of antimicrobial activity by disc diffusion method (Imada et al., 2002). Antimicrobial production was expressed as mg of bioactive molecule per g of biomass (Ellaiah et al., 2002).

Fusion of protoplasts

According to Kieser et al. (2000), protoplast fusion was performed between *Streptomyces* spp. CN207 as a donor strain and naturally chloramphenicol-resistant (cam^R) and *S. coelicolor* M145 as a recipient. 20 µg/ml chloramphenicol (final concentration) was used to select for recombinant strain.

MH agar overlay containing strains testing *S. aureus* and *E. faecalis* were used to select for recombinant strain that possessed antimicrobial activity. Plates were incubated at 37°C for 18 h. Frequency of recombinants colonies was evaluated as the ratio of overproduced strain by the total recombinants (Weaver et al., 2004).

Thin layer chromatography (TLC) analysis

The polyketide antibiotic was isolated from the culture filtrate of the *Streptomyces* spp. CN207 and hybrid *S. coelicolor* PF04 species. The culture filtrates were lyophilised and extracted with ethyl acetate. Then the extracts were redissolved in methanol and analyzed by TLC on silica gel plates (silica gel 60, F_{254} Merck) with ethyl acetate and methanol (100:15) as solvent system and they are visualized with UV light (254 nm) (Caruso et al., 2000). Bands were scraped from the plates, resuspended in a methanol and filtered through wattman paper. Each band was routinely determined by bioassay against an indicator microorganism, as described by Rachev et al. (2003).

Pulse field gel electrophoresis (PFGE) separation and hybridization

PFGE was used to separate large DNA fragments. Mycelial plugs of *Streptomyces* strains were prepared following a standard protocol (Kieser et al., 1992). Restriction endonuclases digest were performed for overnight using *Dra*I, *Ssp*I and *Vsp*I (Promega). PFGE System (Bio-Rad) was used to resolve plug digests using 1.0% pulsed-field-grade agarose gels (Bio-Rad) in 0.5X TBE buffer (50 mM Tris-borate pH 8.0, 0.1 mM EDTA) at 14°C using 6 V cm^{-1} voltage gradient with a 60 s switch time, 120° angle for 24 h. The ladders of linear *S. cerevisiae* concatemers (Bio-Rad) were used as molecular mass markers and gels were stained with ethdium bromide (Thomas et al., 2004). Southern blot hybridization was performed by alkaline transfer of DNA to Hybond N$^+$ membranes (Amersham Life Science, Little Chalfont, and UK) using the manufacturer's protocol. Donor chromosomal DNA digested with *Xba*I was used as probe. Labelling of probe and hybridization was carried out using an ECL direct nucleic acid labelling and detection system kit (Amersham Life Science, Little Chalfont, and UK) following the manufacturer's instruction.

Detection of γ-butyrolactone and bioassay analysis

γ-Butyrolactone was isolated from liquid media SMMS by extracting the culture supernatant of CN207 and *S. lividans* 1326 with ethyl acetate. The solvent was then evaporated and the samples were resuspended in methanol for use in bioassays as described by Takano et al. (2001).

RESULTS AND DISCUSSION

Streptomyces spp. CN207 has attracted attention due to its natural polyketide antibiotic which is active against bacterial and fungal. In fact, *Streptomyces* strains synthesize a broad range of bioactive secondary metabolites, which have made these bacteria a rich source of natural products for the pharmaceutical and agricultural Industries (Chatterjee and Yuan, 2005). Carrying out genetic experiments with protoplast was deemed essential to establish the optimal conditions of protoplast formation and regeneration of a wild type of our local isolate that permits high frequency transfer of a *Streptomyces* spp. CN207 which mediates polyketide synthesis (Fonstein and Haselkorn, 1995).

Optimal conditions for preparation and regeneration of *Streptomyces* spp. CN207 protoplasts

Optimal conditions performed for protoplasts formation and regeneration of *Streptomyces* spp. CN207 are summarized on the Table 1. Protoplast formation was high in

Table 1. Optimal conditions favoured protoplasts formation and regeneration of local *Streptomyces* spp. CN207 and *Streptomyces coelicolor* M145.

Strains	CN207	M145
Culture media	YEME	YEME
Concentration of Glycine	0.5%	0.5%
Concentration of $MgCl_2$	5mM	5mM
Concentration of lysozyme	10mg/ml	10mg/ml
Time of lysozyme treatment	40min	20min
Protoplast formation	3×10^6 protoplast/ml	8×10^7 protoplast/ml
Regeneration media	R2YE	R2YE
Frequency of protoplasts regeneration (%)	1.5	3

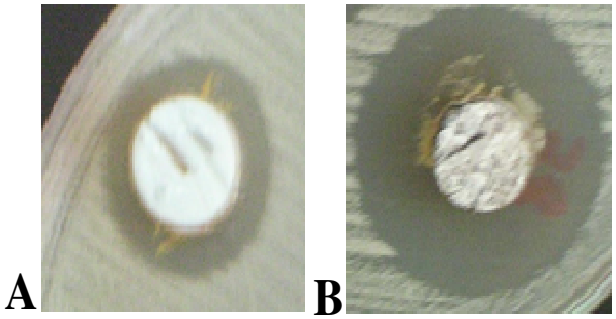

Figure 1. Effect of protoplast formation and regeneration on the antimicrobial production of *Streptomyces* spp. CN207: **A.** Antimicrobial activity by a wild type of *Streptomyces* spp. CN207 against *E. faecalis*. **B.** *Streptomyces* PR-01 antimicrobial activity after regeneration of protoplast of a wild type *Streptomyces* spp. CN207.

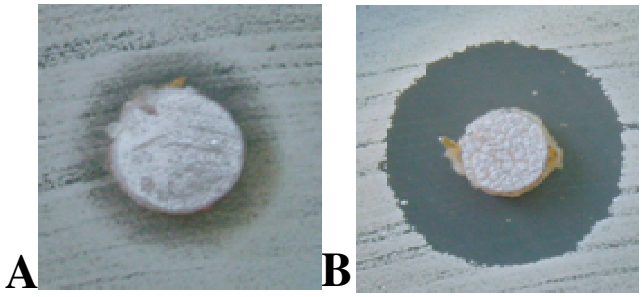

Figure 2. Transfer of the antimicrobial activity after protoplast fusion. **A.** *S. coelicolor* M145 without antimicrobial activity was used as control microorganism. **B.** Antimicrobial activity in solid media of *S. coelicolor* PF04 (after protoplast fusion with *Streptomyces* spp. CN207) against *E. faecalis*.

the early stationary phase and decreased in the late stationary phase. This phenomenon is consistent with the results of Hopwood and Merrick (1977) in *S. acrimycini* and *S. fradiae*. Rodicio et al. (1978) found that protoplast was obtained in young mycelial treated with lysozyme, even when glycine was not added during the growth period. However, in our experiment, the addition of glycine and $MgCl_2$ in YEME media results in much more sensitivity of *Streptomyces* to lysozyme. Regeneration frequencies were 1.5% and 3% for *Streptomyces* spp. CN207 and *S. coelicolor*, respectively. Protoplast regeneration frequency was determined according to the method of Yang and Lei (2001).

Antibiotic activity was studied with twenty five regenerated colonies of *Streptomyces* spp. CN207, using the disc diffusion method (Lorian, 1986). The regenerated colonies were grown in fermentation medium and the antibiotic activity was determined. The antibiotic activity and the CN207 production yield for antibiotic per g biomass obtained for *Streptomyces* PR01 regenerated sample, showed to be about 5 times higher than for the wild strain (Figure 1). These results corroborate those by Ivanova et al. (1992) and Yang and Lei (2001); they reported that protoplasting and protoplast regeneration to improve primary and secondary metabolites has important potential as a future technology for the production of antibiotics.

Evidence for recombinants formed after protoplast fusion and transfer ability for CN207 synthesis

$1x10^6$ protoplast of *Streptomyces* spp. CN207 and *S. coelicolor* M145 were mixed with PEG 4000 and incubated for 2 min. Recipient colonies (*S. coelicolor* M145) initially selected which were able to grow on overlay Muller Hinton (MH) containing 20 µg/ml chloramphenicol and red colour. Another overlay MH agar containing indicator microorganisms to be able to take recipient colonies that acquired the antimicrobial activity. True recombinants were selected after more than three successive purifications. The results showed four recombinant colonies (*S. coelicolor* PF01, PF02, PF03 and PF04) that had antimicrobial activity against indicator microorganisms (Figure 2). Also many of the selective markers, such as Ticarcillin, Cefalotin, Oxacillin, Chloramphenicol, Cefotaxim, etc, were transferred during protoplast fusion (Table 2). As proof of the principal, we demonstrated protoplast fusion that suggests possible transfers of antibiotic CN207 biosynthesis genes by

Table 2. The susceptibilities (zone of inhibition, mm) of *S. coelicolor* PF04, *S. coelicolor* M145 and *Streptomyces* spp. CN207 to the following antibiotics determined by disc diffusion.

Discs of antibiotics	PF04	M145	CN207
Penicillin (6 µg)	6	6	6
Oxacillin (5 µg)	9	6	6
Amoxicillin (25 µg)	22	15	15
Amoxicillin + clavulanic acid (20/10 µg)	26	15	15
Piperacillin (75 µg)	12	20	6
Cefoxitin (30 µg)	6	6	19
Ticarcillin (75 µg)	6	19	6
Cefalotin (30 µg)	13	6	16
Cefoxitin (30 µg)	6	7	19
Cefotaxim (30 µg)	16	6	6
Ceftazidine (30 µg)	6	6	6
Imipenem (10 µg)	33	27	24
Streptomycin (10 µg)	35	32	29
Gentamicin (10 µg)	43	43	45
Tobramycin (10 µg)	38	36	41
Spiramycin (100 µg	19	6	30
Erythromycin (15 µg)	25	23	10
Pristinamycin (15 µg	32	31	26
Norfloxacin (5 µg)	6	6	6
Ciprofloxacin (5 µg)	28	28	29
Ofloxacin (5 µg)	26	24	20
Vancomycin (30 µg)	30	29	32
Teicoplanin (30 µg)	26	24	29
Fosfomycin (50 µg)	6	6	6
Fusidic acid (10 µg)	17	19	25
Chloramphenicol (30 µg)	27	6	29
Thimethoprin – sulfamethoxazole (1.25/23.75 µg)	40	40	30
Fusidic acid (10 µg) Rifamicin (30 µg)	17	19	25
Tetracyclin (30 µg)	26	30	33
Rifamicin (30 µg)	24	21	6

hybrid *S. coelicolor* (Karoonuthaisiri et al., 2005; Ochi et al., 1979). Moreover, Hopwood et al. (1983) and Hotta et al. (1978) proved direct transfer of large DNA fragments between *Streptomyces* species. Also, an iterative protoplast fusion may have resulted in the reassortment of multiple markers between species (Bacher et al., 2002). This is what encouraged us to analyse the recombinant *S. coelicolor* PF04 product for the transfer of production of CN207.

TLC analysis confirmed that cell extract Rf (Retention factor) for *S.coelicolor* PF04 recombinant colonies is identical to that of the wild type (Figure 3). One bioactive region appeared on the chromatogram. Both bands (before and after protoplast fusion) were inhibitory to *S. aureus* and *E. faecalis* in the position. Indeed, the TLC has shown that recombinant PF04 has the same relative location that compared with wild type strain *Streptomyces* spp. CN207 (Lemriss et al., 2003; Macek, 1968).

In addition, to confirm the transfer of antimicrobial CN207 genes, local *Streptomyces* spp. CN207 produced γ-butyrolactone SCB1 and *S. coelicolor* M145 did not. But the recombinant *S.coelicolor* PF04 shows γ-butyrolactone SCB1 production (Figure 4). Results have shown that our local *Streptomyces* spp. CN207 isolate produced a clear antibiotic stimulatory activity when compared to the *S. lividans* 1326 (*Scb A*$^{+}$). Stimulatory activity was visualized as a ring of pigmented mycelial growth surrounding SCB1 extract; blue pigmentation and red colour were very marked on a lawn of *Sreptomyces* M145 (Takano et al.,2005). γ-Butyrolactone is the onset for antibiotic production, and SCB1 directly controls the expression of a pathway-specific regulatory gene in the cryptic type I

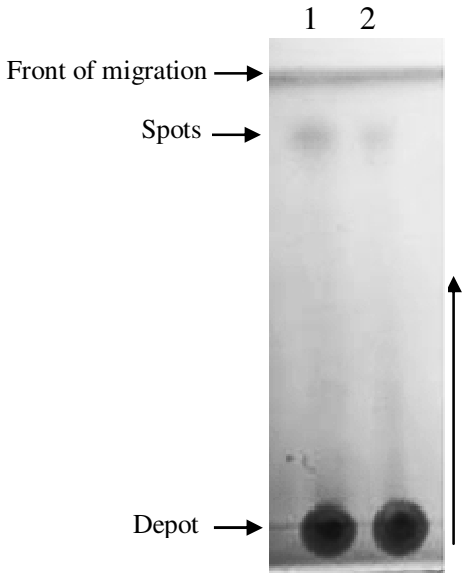

Figure 3. Thin layer chromatography on silica gel plate analysed bioactive compound of *Streptomyces* CN207 (lane 1) and *S. coelicolor* PF04 (lane 2). Long arrow indicates direction of development for chromatogram. Short arrow indicates to spot evident. Solvents used were mixtures of AcOEt: MeOH (100:15). UV 254 nm was used for visualized of chromatogram.

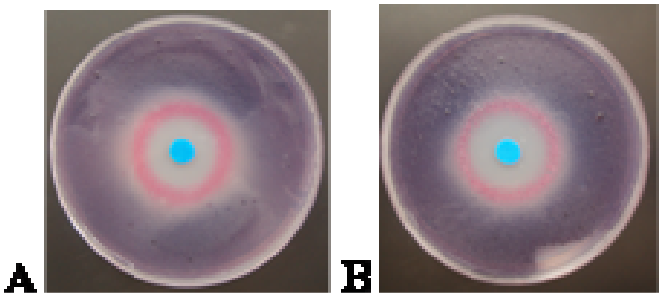

Figure 4. Detection of *scb*A enhances production of the γ-butyrolactone antibiotic stimulatory activity by *S . lividans* TK66 (1326) **(A)** and *Streptomyces* CN207 **(B).** Ethyl acetate extracts from SMMS culture (12) of *S. lividans* and *Streptomyces* CN207 were spotted onto confluent lawns of *S. coelicolor* M145 spores on SMMS and incubated at 30°C for 30 h.

polyketide biosynthetic gene cluster of *S. coelicolor* (Takano et al., 2005; 2001).

PFGE analysis and hybridization

The following strategy was used to deduce the location of the chromosome fragments. Undigested and digested PFGE DNA plugs of parental, donor, recipient and recombinants strains were subjected to PFGE analysis. It is known that molecular mass for *S. coelicolor* M145 chromosomal DNA approximates 8.7 kb (Nitsara et al., 2005) (Figure 5). Results of this experiment showed that

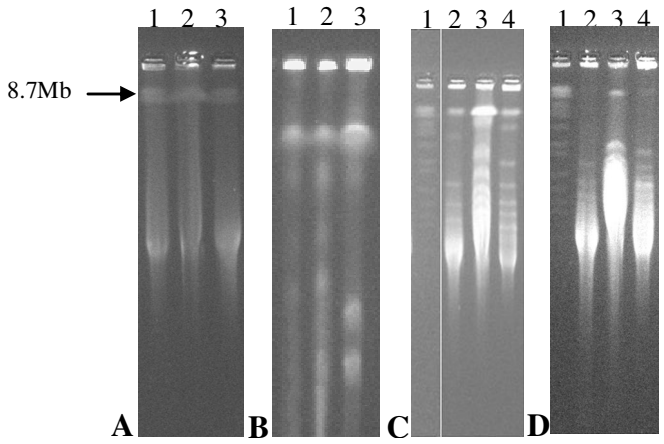

Figure 5. PFGE analysis of undigested chromosome of strain *Streptomyces* CN207 (panel A, lane 1) *S. coelicolor* PF04 (panel A, lane 2) and *S. coelicolor* M145 of ca. 8 Mb in size (panel A, lane 3). The second panel contains DNA chromosome digested with *Dra*I of *Streptomyces* CN207 (panel B, lane 1), *S. coelicolor* PF04 (panel B, lane 2) and *S. coelicolor* M145 (panel B, lane 3). The third panel contains *Ssp*I digestion of DNA chromosome (panel C, lane 1 is *S. cerevisiae* CHEF DNA marker) of *Streptomyces* CN207 (panel C, lane 2), *S. coelicolor* PF04 (panel C, lane 3) and *S. coelicolor* M145 (panel C, lane 4). The fourth panel contains *Vsp*I digestion of DNA chromosome (panel D, lane 1 *S. cerevisiae* CHEF DNA marker) of *Streptomyces* CN207 (panel D, lane 2), *S. coelicolor* PF04 (panel D, lane 3) and *S. coelicolor* M145 (panel D, lane 4). Pulsed-Field Electrophoresis system (Bio-Rad) in 0.5x TBE buffer at 14°C were electrophoresed at 6 V/cm with 60 s switch time, 120° angle for 15 h followed by a 90 s switch time for 9 h for panel B and 3.9 V/cm with 60 s switch time, 120° angle for 20 h for panel A, C and D. The gels were stained with ethidium bromide.

undigested DNA of parental, donor, recipient and recombinants are in the same size (Figure 5A). While the digested DNA showed variability between migrating bands (Figures 5B, C and D), the results showed a discrepancy in DNA fragments between the recipient (*S. coelicolor* M145) and the recombinant (*S. coelicolor* PF04) specially with *Dra*I and *Ssp*I enzymes (Figures 5B and C). *Streptomyces* chromosomes are well known to undergo DNA rearrangements such as deletion and amplification (Adrio and Demain, 2006; Nitsara et al., 2005; Xiuhua et al., 2002). In addition, the results indicate that *Streptomyces* spp. CN207 has no plasmids.

We suggest that the results obtained from the variability between migrating bands are the result of the interspecific gene transfer that took place through protoplast fusion and that the fusants obtained are true recombinants. Also we suggest that a large fragment was transfered from donor to recipient that was responsible for antibiotic production. Protoplast fusion can be used to transfer large (> 100 kb) chromosomal segment from one organism to another without the need for isolation or manipulation of this DNA fragment *in vitro* (Hu et al., 2000). The method does not depend upon the availability of DNA sequence for the entire gene cluster, nor does it require the development of advance genetic tools in the

producing (donor) organism (Pandza et al 1997). Hybridization confirmed the presence of homologous fragments between a wild type *Streptomyces* spp. CN207 with a recombinant *S. coelicolor* PF04, and not *S. coelicolor* M145 (data none shown).

ACKNOWLEDGMENTS

We are very grateful to David A. Hopwood and Nicholas Bird of the John Innes Centre, Norwich, UK for providing *S. lividans* 1326 strain.

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