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Protoplast Fusion and Gene Recombination in the Uncommon Actinomycete *Planobispora rosea* Producing GE2270

Fabrizio Beltrametti†, Daniele Barucco, Roberta Rossi, Enrico Selva, Flavia Marinelli

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Abstract An efficient method for protoplast generation for the uncommon actinomycete *Planobispora rosea*, the producer of the thiazolylpeptide antibiotic GE2270, was developed using a combination of hen egg white lysozyme and *Streptomyces globisporus* mutanolysin. This method converted more than 70% of vegetative mycelium to protoplasts, which were then regenerated with 50% efficiency in an optimized medium. When *P. rosea* protoplasts were efficiently fused, recombination between different antibiotic (streptomycin and gentamicin) resistance markers originated sensitive strains ($str^s gen^s$) at frequencies as high as 18% and double resistant fusants ($str^r gen^r$) at frequencies as high as 29%. Double resistant fusants showed GE2270 productivity intermediate between the productivity of the parental strains. Protoplast generation and fusion in *P. rosea* makes whole genome shuffling feasible as an approach to be used alternately with classical random mutagenesis in industrial strain improvement programs.

Keywords *Planobispora rosea*, protoplast, genome shuffling, GE2270, antibiotic, genetic improvement, actinomycetes

Introduction

Planobispora rosea ATCC 53773 [1] is the producer of the thiazolylpeptide antibiotic GE2270 [2], which acts as a specific inhibitor of bacterial protein synthesis by binding to Elongation Factor Tu. A semi-synthetic derivative of GE2270, named BI-ACNE, has been recently evaluated for clinical development as an anti-acne antibiotic for its activity against *Propionibacterium acnes* [3]. *Planobispora rosea* belongs to the so-called group of uncommon or rare actinomycetes, *i.e.* filamentous actinomycetes other than streptomycetes, which are quite difficult to isolate, cultivate and genetically manipulate [4].

Rare actinomycetes often produce industrially relevant metabolites, but their cost-effective exploitation is usually impeded by the lack of genetic tools, which hinders strain and product improvement. Since mobile genetic elements and conjugation systems are poorly characterized in these microorganisms, a crucial methodology to achieve their transformation by exogenous DNA or to recombine whole genomes (Whole Genome Shuffling–WGS) is based on protoplast manipulation and fusion [5～12]. Protoplast fusion allows the combination of desirable alleles from divergent selection lines into a single strain as a result of the high frequency of intra-strain chromosomal recombination, even when molecular information on the mutated genes is not available. For these reasons, WGS is being increasingly alternated with random mutation cycles (Classical Strain Improvement–CSI) in any breeding program for the improvement of a quantitative character such as antibiotic yield [7, 13～18].

Protoplast preparation and regeneration in *Streptomyces* spp. was originally reported by Okanishi and co-workers

F. Beltrametti (Corresponding author)**, D. Barucco, R. Rossi, E. Selva:** Vicuron Pharmaceuticals S.r.l, via R. Lepetit 34, 21040 Gerenzano (VA) Italy, E-mail: fbeltramett@actygea.it
F. Marinelli: Dipartimento di Biotecnologie e Scienze Molecolari Universita dell'Insubria, Via J. H. Dunant 3, 21100 Varese Italy

† Present address : Actygea S.r.l., via R. Lepetit 34, 21040 Gerenzano (VA) Italy

[19]. The method developed for streptomycetes was then applied with uneven success to *Micromonospora* spp. [20~23], *Brevibacillus* spp. [8], *Amycolatopsis* spp. [24, 25], *Actinoplanes* spp. [26, 27], *Saccharopolyspora erythraea* [28], *Actinomadura verrucosa* [29] and *Kibdelosporangium aridum* [27]. A general conclusion from these studies is that the protocol to be used is species or even strain specific. In other industrially valuable actinomycetes, *ad hoc* techniques have been developed [9] in some cases, but methods with broad applicability and robustness that can be applied for WGS in strain improvement programs are not currently available.

In this report, we describe our methodology for preparing *Planobispora rosea* protoplasts with high efficiency and regenerating these protoplasts so they resume normal filamentous growth. This methodology enabled protoplast fusion to be used to recombine antibiotic resistant genes previously selected in different GE2270 high-producing mutants [30, 31].

Materials and Methods

Strains and Cultural Conditions

Planobispora rosea PR1/5 is a high producing variant induced by MNNG treatment [30, 31]. Strain 162 is a spontaneous PR1/5 streptomycin resistant derivative (str^r); strain RS9 is a spontaneous PR1/5 streptomycin and rifamycin resistant (str^r rif^r) derivative; strains G2 and G67 are spontaneous PR1/5 gentamicin resistant (gen^r) derivatives [30]. Strain characteristics are summarized in Table 1.

Strains were maintained as a lyophilised Master Cell Bank (MCB). A Working Cell Bank (WCB) was prepared from the first generation slant originating from the MCB as already described [32]. Cryo-vials from the WCB were thawed at room temperature and 2 ml were used to inoculate 100 ml of Medium V in 500-ml baffled flasks. Strains were grown for 72~96 hours on a rotary shaker at 200 rpm and 30°C. For protoplast preparation, 10% of the culture was inoculated in 100 ml of Medium VM and growth was allowed for further 96 hours at 30°C and at 200 rpm. Medium V0.1 was routinely used as solid medium. Media composition is described in Table 2.

Protoplasts Formation, Fusion and Regeneration

Dispersed vegetative mycelium, prepared as above described, was centrifuged at 3250×*g*, washed once in P medium [19] and suspended into an equal volume of P medium. Lysozyme, achromopeptidase, proteinase, endoprotease and mutanolysin (SIGMA) for cell wall digestion were dissolved in P medium and added at the final concentrations reported in Table 3. The non-ionic detergent Pluronic (SIGMA) was added at the final concentration of 100 mg/liter. After 16~24 hours incubation with reciprocal shaking, protoplasts were detached from residual mycelium clumps by thoroughly pipetting up and down. Protoplasts were then separated from residual hyphal fragments by filtration through glass wool and eventually through 5 μm durapore membrane filters (MILLIPORE), then centrifuged at 30000×*g*, and re-suspended in fresh P medium. Formation of protoplasts was followed by microscopic observation and they were counted by using a Petroff-Hausser counting chamber and a Zeiss phase-contrast microscope at 400×.

Fusion of protoplasts was performed essentially as suggested by Hopwood *et al.* [12]. In brief, 10^7 protoplasts for each strain involved in the fusion, were pooled, collected by centrifugation and suspended in 0.5 ml of 40% PEG1000 (SIGMA) dissolved in P medium. After 1~3 minutes incubation at room temperature, PEG1000 was diluted to 10% with the addition of 3 volumes of P medium. The protoplast mixture was centrifuged at 10000×*g*, decanted and then re-suspended in fresh P medium.

Regeneration of protoplasts was performed using the overlay technique suggested by Shirahama *et al.* [33]. Plates were seeded by pouring 0.2 ml of protoplast

Table 1 Strains used in this study

Strain	Relevant phenotype
Planobispora rosea PR1/5*	High producing variant of the thiazolylpeptide GE2270, isolated by selection from the parental ATCC 53773 after MNNG treatment
P. rosea 162*	str^r, high GE2270 producer, isolated from PR1/5 by selection on 200 μg/ml streptomycin
P. rosea G67*	gen^r, high GE2270 producer, isolated from PR1/5 by selection on 1 μg/ml gentamicin
P. rosea G2*	gen^r, high GE2270 producer, isolated from PR1/5 by selection on 1 μg/ml gentamicin
P. rosea RS9*	str^r, rif^r, high GE2270 producer, isolated from 162 by selection on 100 μg/ml rifamycin

* Reference 30.

Table 2 Medium composition

Component	Medium V	Medium VM	Medium V0.1	M3	Medium VMS0.1
Soluble starch (DIFCO) (g/liter)	24	24	2.4	2.4	2.4
Dextrose (g/liter)	1	1	0.1	0.1	0.1
Meat extract (g/liter)	3	3	0.3	0.3	0.3
Yeast extract (g/liter)	5	5	0.5	0.5	0.5
Triptose (g/liter)	5	5	0.5	0.5	0.5
Agar (g/liter)	0	0	15	15	0
Low melting point agarose (g/liter)	0	0	0	0	4
L-Proline (g/liter)	0	3.5	0	3.5	3.5
Sucrose (g/liter)	0	103	0	80	103
$MgCl_2$ (mmol/liter)	0	0	0	10	0
$CaCl_2$ (mmol/liter)	0	0	0	50	0

Media were prepared in de-ionized water and pH was adjusted to 7.2 after sterilization.

suspension on M3 agar and then overlaid with 4.0 ml of melted, pre-cooled (about 30°C) Medium VMS0.1 (Table 2). Plates were incubated at 28°C for 20～30 days. Since protoplasts hardly regenerated on media supplemented with antibiotics, the efficiency of recombination among antibiotic resistant strains was determined by replica-plating the regenerated colonies onto Medium V0.1 plates and onto Medium V0.1 plates supplemented with 200 mg/liter streptomycin, 100 mg/liter rifamycin or 1.0 mg/liter gentamicin.

Liquid Media Fermentation

For antibiotic production, growth conditions in liquid media were essentially as described by Gastaldo and Marinelli [31]. Strains were inoculated in 100 ml of D/Seed vegetative medium in 500-ml baffled flasks. After 72-hour growth on a rotary shaker at 200 rpm and 28°C, 10% of the culture was inoculated in 100 ml of Medium C and fermentation was allowed for 7 days at 28°C and 200 rpm. Production was estimated by HPLC as described below and productivities reported are relative to the PR1/5 strain.

GE2270 Extraction and Analysis

Samples were collected from fermentation flasks and processed for GE2270 extraction by mixing one volume of whole culture with two volumes of acetonitrile and vortexing at room temperature for 30 seconds. Samples were centrifuged at 3250×*g* for 10 minutes and the supernatant was analysed by HPLC using an analytical 5 μm particle size Ultrasphere ODS (Beckman) column (4.6×250 mm) eluted at 1.5 ml/minute flow rate with a 20 minutes linear gradient from 45% to 75% (v/v) of Phase B. Phase A was 20 mmol/liter NaH_2PO_4 : CH_3CN 9 : 1 (v/v) and Phase B was 20 mmol/liter NaH_2PO_4 : CH_3CN 3 : 7 (v/v) mixture. The chromatography was performed with a Hewlett Packard model 1100 HPLC system and detection was at 310 nm. As standard for antibiotic titre determination, a sample of GE2270 antibiotic was used. GE2270 production was calculated as already described [31].

Selection Criteria and Characterization of Resistant Mutants

GE2270 production in the parental populations was estimated by fermenting *ca.* 50 independent clones in triplicate. Clones were distributed in classes on the bases of their average GE2270 productivity, giving a normal distribution. Production of GE2270 double resistant clones was estimated by fermenting at least 40 independent mutants in triplicate, and showed a peculiar distribution for each combination of the resistance markers. The Standard Deviation (SD) among the replicas did not exceed 10%. We define as high producers those mutants producing more than the highest GE2270 producing clone among parental controls [30].

Results and Discussion

Protoplast Preparation

Reported methods for protoplast production are based on lysozyme treatment combined with other cell wall hydrolysing enzymes [9～12, 27, 33～35]. The application of these protocols to members of *Streptomyces* gave a vast range of efficiencies in protoplast formation varying within species and strains. When we tried to apply the known procedures to the *Planobispora rosea* strains described in Table 1, the first obstacle was that none of the described

media [9, 36] designed for generating biomass for protoplast formation, supported *Planobispora rosea* growth. To overcome this problem, we screened a number of rich media previously used for *Planobispora rosea* biomass production (Beltrametti *et al.* unpublished results) and supplemented them with different concentrations of sucrose and proline to acclimate *Planobispora rosea* to the components of the hyper tonic buffer (P medium) used for the subsequent cell wall digestion [37, 38]. We, in fact, noticed that in the case of growth medium not supplemented with sucrose and proline (the latter acts as osmo-protectant [39]), mycelium suspension into the digestion buffer led to the formation of compact tough pellets, which were difficult to convert into protoplasts. Sub-cultivation of *Planobispora rosea* in a starch-based medium containing sucrose and proline (Medium VM, Table 2) gave the best compromise between biomass production and protoplast yield and thus it was selected for the following experimentation.

When different combinations of commercially available lysozyme (hen egg white lysozyme or HEWL) (5.0 mg/ml) and other enzymes were tested, only the one containing mutanolysin (lysozyme from *Streptomyces globisporus* or ML1) led to a significant production of protoplasts (Table 3). It has been reported that lysozymes from diverse biological sources show a different specificity for peptidoglycan preparations from a number of Gram-negative and Gram-positive bacteria [40]. *Planobispora rosea* peptidoglycan was better cleaved by a *Streptomyces* enzyme. Addition of proteolytic enzymes was not helpful (Table 3). Optimal concentration of mutanolysin to convert mycelium to stable protoplasts was at 0.018 mg/ml. A generalized positive effect on protoplast formation was observed by the addition of the non-ionic detergent Pluronic at a concentration of 100 mg/liter. Likely, Pluronic protected protoplasts from hydrodynamic damage as already reported for eukaryotic cells [41, 42]. The efficiency of protoplast formation was assayed by microscopic enumeration at different times of incubation in the digestion solution. In contrast to the fast protoplast formation observed in *Streptomyces* spp. (*ca*.15 minutes) [43], maximum protoplast yield ($10^7/10^8$ protoplasts per ml of culture) were achieved with *Planobispora rosea* after 16 to 24 hours of incubation. This results in approximately 70% conversion of the mycelium to protoplasts. No appreciable difference was observed depending on whether the mycelium was sampled during the exponential or the stationary phase of growth (data not shown).

Protoplast Regeneration

Regenerating mycelium from protoplasts in *Planobispora rosea* has been attempted by using a series of hypertonic synthetic agar media reported in the literature [9, 19, 33, 43]. None of them supported *Planobispora rosea* growth. We used 1/10 diluted Medium V (Medium V0.1, Table 2) and its hypertonic version (VM0.1, Table 2) supplemented with 103 g/liter sucrose and 3.5 g/liter proline. In this last condition, regenerating colonies were observed after 15～30 days of incubation at 30°C. To distinguish colonies regenerated from protoplasts from those growing from residual contaminating hyphal fragments, samples from different protoplast preparations were diluted in P medium and then plated both on non-permissive Medium V0.1 and on permissive Medium VM0.1. Fig. 1 shows that short time incubation (1, 3, 6 hours) in protoplasting solution resulted in high contamination by whole cells (despite filtration through glass wool), since a comparable number of colonies grew in both the media. After 24-hour treatment, colonies were detectable only in hypertonic medium. In these conditions, 0.1% of the protoplasts counted by microscope regenerated to produce colonies (Fig. 1). No appreciable difference in regeneration efficiency was observed if protoplasts originated from hyphae in the exponential or in the stationary phase of growth (data not shown).

To improve the regeneration efficiency of 24-hours protoplasts, Medium VM0.1 was modified. We observed that reducing sucrose concentration from 103 to 80 g/liter

Table 3 Effect of digestion enzymes combined with 5.0 mg/liter lysozyme (HEWL) on protoplast formation

Enzyme	Concentration range (mg/ml)	Protoplast formation	Number of protoplasts per ml in 24 hours	Reference or source
None	—	$<10^5$		
Mutanolysin	0.001～25	++	1×10^7～1×10^8	Reference 44
Achromopeptidase	5～25	—	$<10^5$	Reference 9
Proteinase	5～25	—	$<10^5$	This work
Endoprotease	5～25	—	$<10^5$	This work

—: None or scarce; ++: Good.

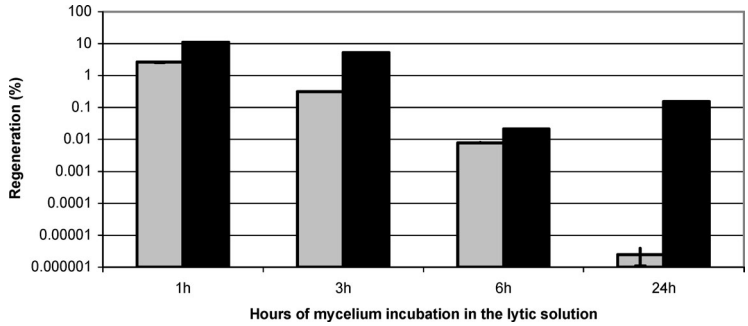

Fig. 1 Protoplast regeneration from cultures incubated for different times with digestion enzymes.

Protoplasts were plated on Medium V0.1 (grey bars) and Medium VM0.1 (black bars). Colonies growing on Medium V0.1 originated from residual hyphal fragments, while protoplasts regenerated only on Medium VM0.1. Incomplete treatment with digestion enzymes originated a high proportion of contaminating hyphae.

improved the growth rate and the dimension of regenerating colonies (data not shown), although the final number of regenerating colonies did not change. Following Okanishi and co-workers [19], regeneration medium was modified by searching for the optimal concentrations of $CaCl_2$, $MgCl_2$ and phosphate. $CaCl_2$ addition was found to exert a positive effect, which was antagonized by phosphate buffer. $MgCl_2$ showed a limited impact on protoplast regeneration (data not shown). The best combination of the supplemented micronutrients was 10 mmol/liter $MgCl_2$, 50 mmol/liter $CaCl_2$ and no phosphate. In this condition (M3 medium, Table 2) 30～50% of the protoplasts observed by microscope were regenerated and formed colonies. Previously reported efficiencies of protoplast regeneration in rare actinomycetes ranged from 1 to 37%, *i.e.* 15% in *Actinomadura verrucospora* [29], 4.0% in *Micromonospora echinospora* [23], 30% in *Micromonospora rosaria* [21], 36.7% in *Micromonospora purpurea* [9], 6.6% in *Saccharopolyspora eritharea* [28], and 5.0% in *Amycolatopsis orientalis* [24]. Protoplast regeneration efficiency was up to 90% in *Streptomyces* spp. [33].

Recombination of Resistance Genes by Protoplast Fusion

Protoplast fusion has been demonstrated as a powerful tool for the WGS when alternated with classical random mutagenesis in the improvement of antibiotic producing fungi and actinomycetes [16～18]. A general requirement for the assessment of this methodology is the possibility to calculate recombination frequency between genomes carrying different selectable markers. To this purpose, protoplasts of mutants of *Planobispora rosea* previously selected as resistant to streptomycin (162 and RS9) or gentamicin (G67 and G2) [30] (see Table 1 for strain description), were fused in the following combinations: 162×G2, 162×G67, RS9×G2 and RS9×G67 (Fig. 2). As previously demonstrated the mutations selected in the mutants 162, RS9, G2 and G67 conferred resistance to the antibiotic and also determined an increase in production of GE2270 [30]. As a consequence, we expected that the combination of resistances also determined variations in the GE2270 production.

Protoplast fusion was performed according to Hopwood *et al.* [12] using PEG1000 as the aggregating agent. Protoplasts regenerated poorly on M3 medium supplemented with antibiotics. Thus, the efficiency of recombination between the resistant strains was determined by replica-plating regenerated colonies onto plates supplemented with streptomycin and gentamicin, streptomycin, gentamicin and on Medium V0.1 without antibiotics. The percentage of str^rgen^r, str^sgen^s, str^sgen^r, str^rgen^s clones was determined. Un-fused protoplasts from the parental strains were used as controls. Fig. 2 shows that the frequency of double sensitive (str^sgen^s) clones (which are the leading indicators of genome recombination—see below) originating from protoplast fusion ranged from 3.6 to 18% of the population, whereas the spontaneous double sensitive colonies in the un-fused populations never exceeded the 0.01%. The frequency of double-resistant clones (str^rgen^r) ranged instead between 8.9 and 29%. Discrepancies between the population of double sensitive and double resistant clones in favour of the phenotype str^rgen^r was probably determined by spontaneous mutations determining str^r at high frequency (as already reported for G2 and G67 strains [30]) and/or by the fact that, in myceliar microorganisms, different genomes (*i.e.* str^rgen^s and str^sgen^r) can coexist determining a str^rgen^r phenotype. Efficiency of protoplast fusion and genome recombination varied depending on which strains were crossed, but both the events occurred at significantly higher frequency in comparison to spontaneous mutation (Fig. 2).

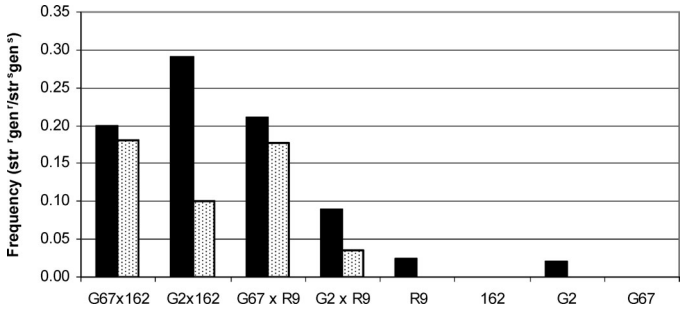

Fig. 2 Frequency of double resistance (str^{r}gen^r—black bars) and double sensitive (str^{s}gen^s— dotted bars) clones originating after bi-parental protoplast fusion.

Resistant clones were selected by replica on plates supplemented with streptomycin and gentamicin. In sensitive clones the gen^r and str^r of the parental clones was excluded by replica on str and gen plates. Data concerning productivity of the fusing strains are described in detail in the text.

The recombination of mutants G67×162 yielded double resistant fusants (str^{r}gen^r) showing a GE2270 productivity (relative to the PR1/5 strain productivity [30]) intermediate between the parental strains (1.33 *vs.* 1.10 fold for strain 162 and 1.5 for strain G67). Similar results were obtained from the fusion G2×162 (1.39 *vs.* 1.1 fold for strain 162 and 1.44 for strain G2). Double resistant fusants from recombination of G67×RS9 were typically grouped in two classes: fusants producing less GE2270 than G67 (*ca.* 1.33 fold) and those giving an intermediate productivity between the two parental strains (1.60 *vs.* 1.50 fold for strain G67 and 1.77 for strain RS9). This two classes distribution was also obtained from the G2×RS9 fusion and it is explained by the presence in RS9 of two distinct mutations (conferring streptomycin and rifamycin resistance), whose only one was selected (the one conferring streptomycin resistance). Overall, these GE2270 production data from fused and regenerated colonies confirm our previous observations on sequentially mutated str^{r}gen^r strains showing intermediate productivity between the high producer parental strains resistant either to streptomycin or to gentamicin [30]. This proves that once proptoplasts are efficiently produced, fused and regenerated, WGS in actinomycetes may replace labour intensive procedures of recombining different mutations independently achieved by random mutation. Sister-crossing and back-crossing approaches became as feasible as in eucariotyc cells capable of sexual mating [7, 13～18].

Further work is ongoing to apply this methodology to other uncommon actinomycetes producing valuable antibiotics such as *Nonomuraea*, *Microtetraspora*, *Planomonospora* and *Streptosporangium* spp. Preliminary results show that these protocols of protoplast formation and fusion are broadly applicable to other actinomycetes, whereas the more critical and strain-specific phase is the protoplast regeneration, which requires the development of *ad hoc* regeneration medium (F. Beltrametti, unpublished results).

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