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ORIGINAL PAPER

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Antibiotic production improvement in the rare actinomycete *Planobispora rosea* by selection of mutants resistant to the Aminoglycosides Streptomycin and Gentamycin and to Rifamycin

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Abstract During a strain improvement program, spontaneous mutants with single or combined resistance to streptomycin (Str^r), gentamycin (Gen^r) or rifamycin (Rif^r) were selected from the industrial strain of *Planobispora rosea*, which is the producer of thiazolylpeptide GE2270. Among the mutants resistant to each single antibiotic, higher producers occurred more frequently (60%) among Gen^r than in Rif^r (10%) and Str^r (24%) populations. Two Gen^r mutants showed up to 1.5-fold improvement in GE2270 production while single resistant mutants Str^r and Rif^r produced slightly more than the parental strains. The combination of Str^r and Rif^r in the same strain improved GE2270 yield up to 1.7-fold. Finally, a higher GE2270 producing strain (1.8-fold improvement with respect to the parental strain) was selected among those mutants with triple resistance to streptomycin, rifamycin and gentamycin. A hierarchical increase in aerial mycelium and spore formation was observed which paralleled GE2270 production improvement.

Keywords GE2270 · Strain improvement · *Planobispora rosea* · Resistance · Sporification

Introduction

Wild type strains typically produce limited quantities of secondary metabolites and thus during a drug development process, there is a need of iterative rounds of strain improvement to supply their bioactive products in significant quantities. In this frame, the application of modern techniques of direct genome manipulation frequently encounters serious impediments due to limited insight into the genetics, physiology and biochemistry of the producer organism, which indeed often belongs to a poorly studied group. A traditional approach of classic strain improvement is thus generally pursued: the introduction of random mutations followed by massive screening searching for higher-producing mutants [1, 24]. In this approach, techniques that efficiently select mutants with potential high-producing traits (prescreening) are fundamental to reducing efforts that would otherwise be spent in the subsequent screening process.

Spontaneous mutants resistant to streptomycin (Str^r) and/or rifamycin (Rif^r) and/or gentamycin (Gen^r) of *Streptomyces* spp. are reported in the literature to overproduce secondary metabolites [7, 9, 19]. The frequency of higher producers among the resistant *Streptomyces* spp. populations ranged from 3 to 50% [7, 9, 19]. Although the mechanism linking antibiotic resistance to overproduction of secondary metabolites has not yet been completely elucidated, it has been proposed that modulation of the translational apparatus by induction of *str* and *gen* mutations and of transcription by *rif* mutations may function cooperatively to increase productivity through interaction with the stringent response mechanism [20]. Data concerning few model antibiotics are reported [7, 9, 10, 17, 25] and the effectiveness of this method on one industrial *Streptomyces* strain has been recently proved [20].

Planobispora is an uncommon genus of actinomycetes of the family *Streptosporangiaceae*, comprising two species only [22]. One of the two species, namely *Planobispora rosea* ATCC 53773, is the producer of antibiotic GE2270 [6, 11, 18]. This antibiotic is a potent inhibitor of protein synthesis in Gram-positive bacteria [6, 18] and it is in clinical trials as semi-synthetic derivative BI-K0376 targeted against *Propionibacterium acnes* [11].

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In this paper, we describe how the isolation of mutants resistant to streptomycin, gentamycin and rifamycin allowed the rapid increase of GE2270 production in this uncommon actinomycete.

Materials and methods

Strains and cultural conditions

The strains used in this study are described in Table 1. *P. rosea* PR1/5 was isolated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Fluka, Buchs, Switzerland) treatment from the natural variant PR9/1 [5]. Spontaneous resistant mutants of strain PR1/5 (and selected derivatives) arose with frequencies between 10^{-7}–10^{-9} and were selected by spreading sonicated hyphae, prepared according to Beltrametti et al. [2], on plates supplemented with 200 mg/l streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 mg/l rifamycin (Sigma-Aldrich) or 1 mg/l gentamycin (Sigma-Aldrich). These antibiotic concentrations were 5–10-fold the minimal inhibitory concentrations (MICs) for PR1/5 strain, determined by agar dilution method [15]. After incubation for 30 days at 28°C, resistant clones were replicated with a toothpick on plates containing the required antibiotics and then processed for fermentation.

For aerial mycelium and spore formation, strain PR1/5 and resistant derivatives were seeded on Soil Agar [23]. Plates were incubated for 15 days at 30°C. Microscopic observations were performed with a Zeiss Standard 25 microscope and pictures were taken with a DP10 Olympus digital camera.

Liquid media fermentation

Growth conditions in liquid media were essentially as described by Gastaldo and Marinelli [5]. Strains were inoculated in 100 ml of D/Seed vegetative medium (gram/liter: soluble starch (Cerestar, Mechelen, Belgium), 20; polypeptone (A. Costantino & C. S.p.a., Favria, Italy), 5; Meat extract (A. Costantino & C. S.p.a.), 2; yeast extract (A. Costantino & C. S.p.a.), 3; soybean meal (Cargill Inc., Cedar Rapids, IA, USA), 2; $CaCO_3$ (Carlo Erba, Milan, Italy), 1; pH 7, deionized water up to 1 liter) in 500-ml baffled flasks. Strains were grown for 72 hours on a rotary shaker at 200 rpm and 28°C. Ten percentage of the above described culture was inoculated in 100 ml of Medium C (gram/liter: soluble starch, 35; glucose (J. T. Baker, Phillipsburg, NJ, USA), 10; hydrolized casein (A. Costantino & C. S.p.a.), 5; meat extract, 3.5; yeast extract, 8; soybean meal, 3.5; $CaCO_3$, 2; Vitamin B12 (Merck, Darmstadt, Germany), 0.005; pH 7.2, deionized water up to 1 liter) and fermentation was allowed for 7 days at 28°C with shaking at 200 rpm. Production was estimated by HPLC as described below.

As previously reported, the addition of Vitamin B12 to the fermentation medium directed GE2270 complex production toward the selective synthesis of factor A [5]. Thus, data herein reported refer to GE2270 factor A production.

GE2270 extraction and analysis

Samples were collected from fermentation flasks and processed for GE2270 extraction by mixing one volume of the whole culture with two volumes of acetonitrile and vortexing at room temperature for 30 s. Samples were centrifuged at 3,250 *g* for 10 min 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/min flow rate with a 20 min linear gradient from 45 to 75% (v/v) of Phase B. Phase A was 20 mM NaH_2PO_4: CH_3CN 9:1 (v/v) and Phase B was 20 mM NaH_2PO_4: CH_3CN 3:7 (v/v) mixture. The chromatography was performed with a Hewlett Packard mod 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 [5].

Selection criteria and characterization of resistant mutants

Production of GE2270 in the parental populations was estimated by fermenting ca. 50 independent clones in

Table 1 Selection of single and combined resistance mutants

Parental strain and resistance phenotype	Antibiotic used for selection	Number of fermented mutants	Frequency of mutants	Improved mutants (%)[a]	Mutant selected for further improvement and resistance phenotype	Relative GE 2270 improvement [b] (±SD)
PR1/5	Streptomycin	290	7.3×10^{-7}	24	162 (Str^r)	1.11 ± 0.08
PR1/5	Rifamycin	153	7.3×10^{-8}	10	72 (Rif^r)	1.10 ± 0.09
PR1/5	Gentamycin	283	1.9×10^{-7}	60	G2 / G67 (Gen^r)	1.52 ± 0.11
162 (Str^r)	Rifamycin	40	7.1×10^{-8}	13	RS9 (Str^r, Rif^r)	1.70 ± 0.14
162 (Str^r)	Gentamycin	42	2.5×10^{-8}	75	GS15 (Str^r, Gen^r)	1.25 ± 0.04
G2 (Gen^r)	Rifamycin	42	3.2×10^{-7}	0	//	//
GS15(Str^r Gen^r)	Rifamycin	123	1.5×10^{-6}	44	GSR39 GSR77 (Str^r, Gen^r, Rif^r)	1.82 ± 0.02

[a]Relative to the parental strain
[b]Relative to the average production of strain PR1/5

triplicate. The standard deviation (SD) of the three GE2270 production values obtained did not exceed 10%. Clones were distributed in classes on the bases of their average GE2270 productivity giving a normal distribution. Production of GE2270 by resistant populations was estimated by fermenting at least 40 independent mutants in triplicate (Table 1) and showed a characteristic distribution for each resistance marker or marker combination. The SD of the three GE2270 production values obtained did not exceed 10%. We define as high producers those mutants producing more than the highest GE2270 producing clone among parental controls.

Results and discussion

Construction of antibiotic resistant mutants

Initially, single resistance mutants were selected from strain PR1/5. A set of resistant colonies were randomly chosen and fermented for GE2270 production according to Gastaldo and Marinelli [5] (Table 1). Clones were grouped in classes according to their GE2270 productivity resulting in a characteristic distribution for each resistance marker (Fig. 1a). Antibiotic production levels were distributed over a broader range in comparison to the parental strain PR1/5. Figure 1a shows that 60, 10 and 24% of the Gen^r, Rif^r and Str^r populations respectively, had an improved GE2270 production. From these high producing populations, selected clones confirmed improved antibiotic production in repeated fermentations. As summarized in Table 1, Gen^r mutants G2 and G67 showed 1.5 times the average production of strain PR1/5. Str^r mutant 162 and Rif^r mutant 72, were also selected and produced 1.1 times versus the control.

The Gen^r G67 and G2 mutants were found cross-resistant to streptomycin and no further improvement was achievable plating them on rifamycin. Cross resistance between the Str^r and Gen^r was instead absent in the Str^r mutant 162 (data not shown) which indeed became the parental strain for further improvements.

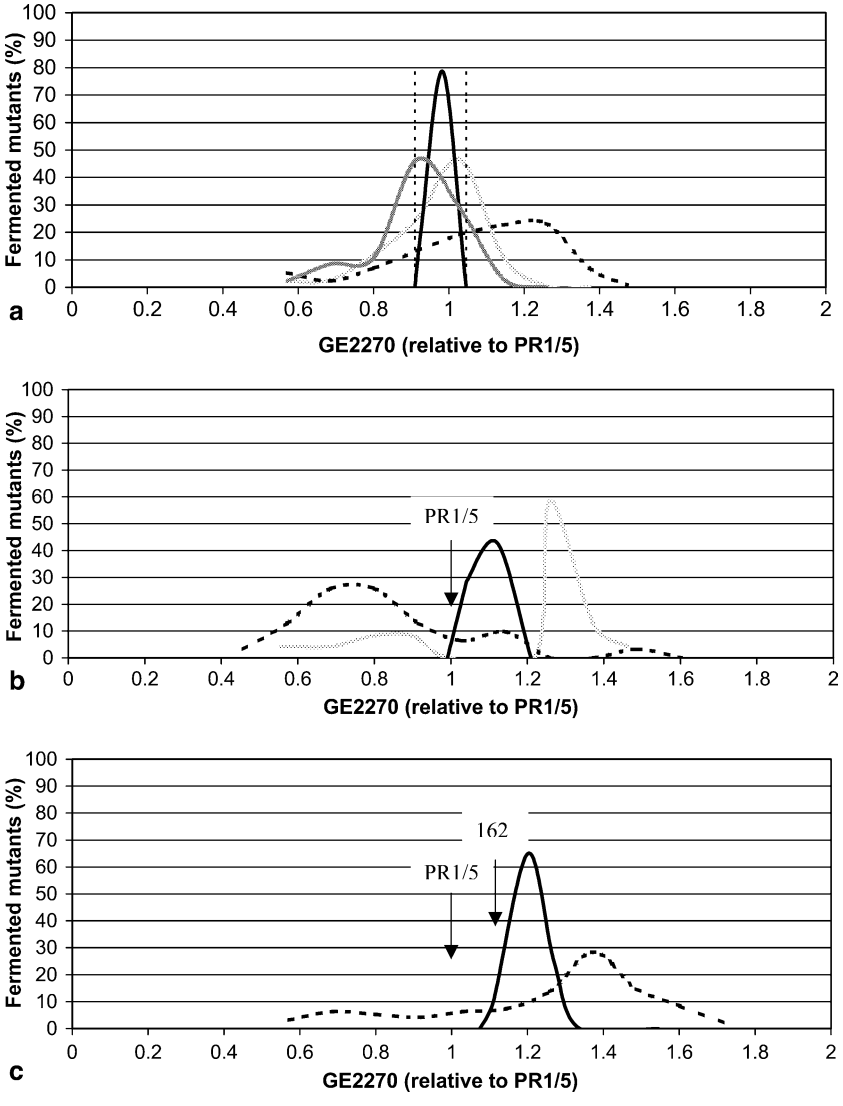

Fig. 1 **a** Distribution of GE2270 production in mutants resistant to streptomycin (*light gray line*), gentamycin (*dashed line*), rifamycin (*gray line*) versus strain PR1/5 (*continuous line*). *Vertical dashed lines* define the lowest and the highest GE2270 producing clone in PR1/5 population. **b** Distribution of GE2270 production in strain 162 (Str^r) mutants selected on rifamycin (*dashed line*) and gentamycin (*light gray line*) versus strain 162 (*continuous line*). **c** Distribution of GE2270 production in strain GS15 (Str^r,Gen^r) mutants selected on rifamycin (*dashed line*) versus strain GS15 (*continuous line*). Average GE2270 production in strain PR1/5 and 162 are indicated by *arrows*

Spontaneous mutants of Str^r strain 162 were selected on 100 μg/ml rifamycin or 1 μg/ml gentamycin (Table 1) and again their antibiotic production was found distributed on a broader range with respect to the parental strain (Fig. 1b). Rif^r mutants resulted distributed in three classes according to their production level, with 13% of the mutants producing more than the parental strain. From this group of double resistant mutants, clone RS9 was isolated which confirmed improved production in repeated fermentations (ca. 1.7-fold vs. the PR1/5 strain).

Figure 1b shows also that the majority (75%) of the Gen^r mutants from Str^r strain 162 produced more than the parental strain. From this round of selection, the Gen^r Str^r clone GS15 was selected after repeated fermentations (1.2-fold production with respect to strain PR1/5) and was plated on rifamycin to select triple resistance mutants. As shown in Fig. 1c, 44% of triple resistant mutants showed improved production and among them strains GSR39 and GSR77 (Str^r, Gen^r, Rif^r) were finally selected because of their capability to consistently produce 1.8 times more than the PR1/5 strain (Table 1). Figure 2a summarizes the genealogy of the mutants described above. Mutant GSR39 was scaled up to 15 l tank and confirmed the GE2270 production observed in flask (data not shown). This mutant was periodically re-selected on the required antibiotics and did not show loss of resistance or fall in GE2270

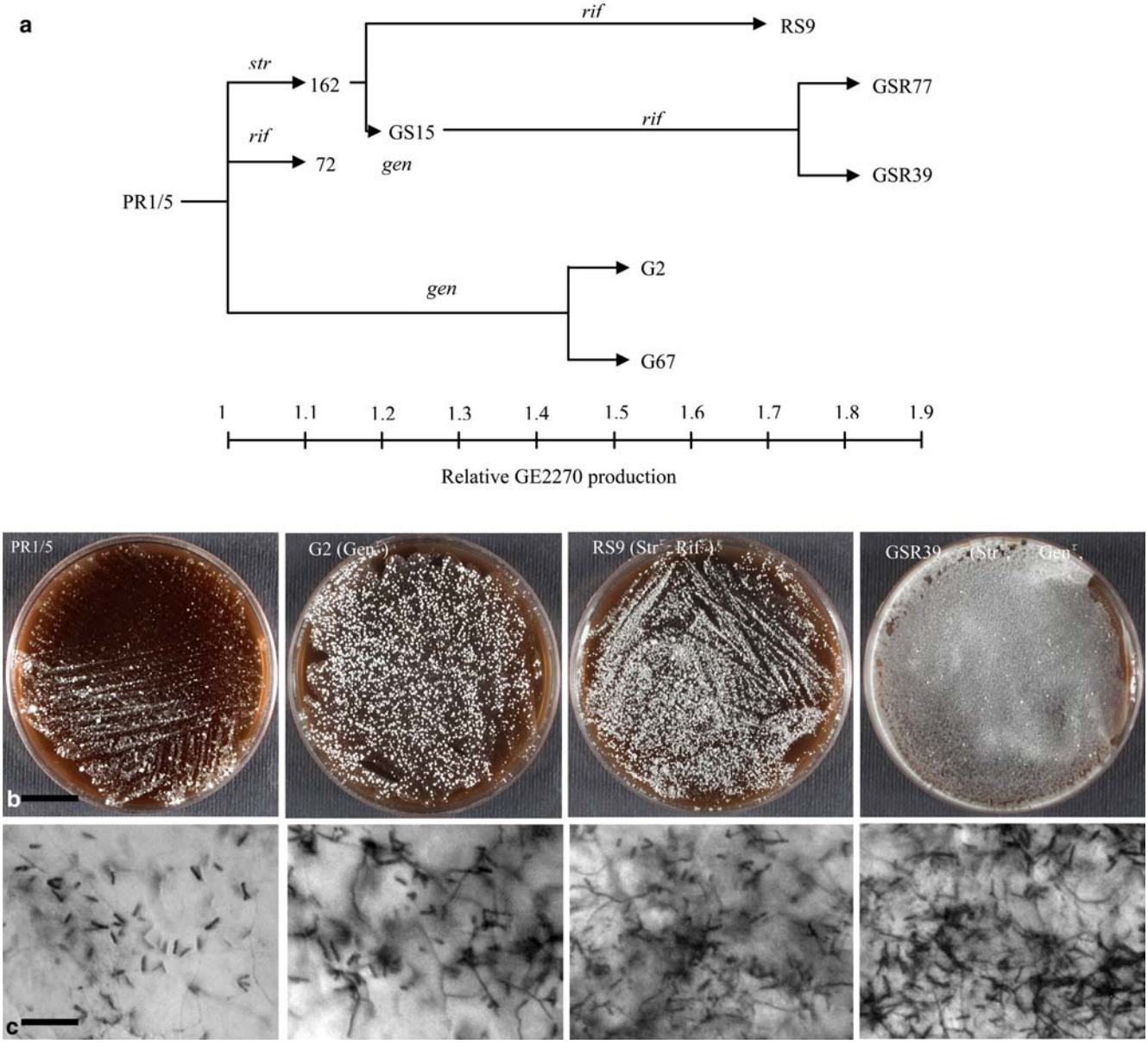

Fig. 2 **a** Schematic representation of the genealogy of high producers. Production in the mutants is relative to the average value in strain PR1/5. **b** Production of aerial mycelium and **c** sporangia by parental PR1/5 and mutants of *P. rosea*. Sporangia are visible as black rods in the microscopic preparation (400× magnification). *Bar intervals* are 1 cm (**b**) and 15 μm (**c**)

production. This confirmed the suitability of this strain for industrial GE2270 production.

Analysis of sporulation in resistant mutants

It is reported in literature that Str^r, Rif^r and Gen^r mutations resulting in an improved production of antibiotics were associated with the differentiation of abundant aerial mycelium and spores in *S. coelicolor* and *S. albus* [9, 19, 20]. In *P. rosea*, sporangia are born in bundles on sporangiophores branched from the aerial hyphae [22] and each sporangium is visible as a short rod. We seeded strain PR1/5 and resistant derivatives on Soil Agar [23] and aerial mycelium had the appearance of a white fluffy hyphal mass on the surface of the plates (Fig. 2b). Resistant mutants G2, RS9 and GSR39, with an antibiotic production significantly improved, showed an increased formation of sporangia-bearing aerial mycelium (Fig. 2b, c) confirming the phenotype reported for *Streptomyces* spp. mutants [9, 19, 20].

In conclusion, we found that part of spontaneous Str^r, Rif^r and Gen^r populations of *P. rosea* produced high amounts of GE2270. The combination of Str^r and Rif^r mutations in double mutants and of Str^r, Rif^r and Gen^r mutations in triple mutants led to a further increase in antibiotic production (Fig. 2a).

In *S. coelicolor* Str^r mutants with an improved antibiotic production, the mutations were located in the *rpsL* gene encoding for ribosomal protein S12 [7]. In Rif^r mutants, mutations are typically located in the *rpoB* gene encoding for RNA polymerase [12]. High production of secondary metabolites in these mutants is considered due to overexpression of the differentiation phase proteins, which in turn can be determined by an increase in either transcription [14, 25] or translation [20].

Gentamycin resistance is reported in *Escherichia coli*, to arise from *rplF* gene, encoding for the ribosomal L6 protein [3, 13, 20] or from mutations on the 23S rRNA 2660 loop region [16, 21]. *E. coli* mutants in *rplF* or in the 23S rRNA 2660 loop region showed gentamycin resistance and resistance to other aminoglycoside antibiotics, including streptomycin [8, 16]. We found that *P. rosea* Gen^r strains G2 and G67, were cross-resistant to streptomycin (data not shown) and in this aspect they resemble *E. coli* mutants. Notwithstanding the higher productivity of these two mutants, no further improvement was achievable from them by selection on streptomycin and rifamycin. Cross-resistance between the Str^r and Gen^r was instead absent in the Str^r mutant 162 (data not shown) which indeed became the parental strain for further improvements.

It would be interesting to study the genetics and the physiology of the improved *P. rosea* strains to get deeper insight on how Str^r, Rif^r and Gen^r cooperatively influence the production of antibiotic GE2270. GE2270 is a highly modified thiazolylpeptide whose biosynthesis [4] is different from the other antibiotic classes already improved with this approach. Considering this and the fact that *P. rosea* is an uncommon actinomycete, these results further support previous reports showing that a pre-screening technique based on selection of *str*, *rif* and *gen* mutations may have broad application in industrial strain improvement.

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