Metabolic Engineering 26 (2014) 1–16

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Comparative genomics revealed key molecular targets to rapidly convert a reference rifamycin-producing bacterial strain into an overproducer by genetic engineering

Clelia Peano [a,1], Fabrizio Damiano [b,1], Mattia Forcato [c], Alessandro Pietrelli [a], Carla Palumbo [b], Giorgio Corti [a,2], Luisa Siculella [b], Fabio Fuligni [a,3], Guidantonio Malagoli Tagliazucchi [c], Giuseppe Egidio De Benedetto [d], Silvio Bicciato [c], Gianluca De Bellis [a], Pietro Alifano [b,*]

[a] Institute of Biomedical Technologies, National Research Council, Segrate, Milan 20090, Italy
[b] Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Lecce 73100, Italy
[c] Center for Genome Research, Department of Life Sciences, University of Modena and Reggio Emilia, Modena 40125, Italy
[d] Laboratory of Analytical and Isotopic Mass Spectrometry, Department of Cultural Heritage, University of Salento, Lecce 73100, Italy

ARTICLE INFO

Article history:
Received 28 March 2014
Received in revised form
8 August 2014
Accepted 10 August 2014
Available online 19 August 2014

Keywords:
Amycolatopsis mediterranei
Rifamycin
Methylmalonyl-CoA mutase
Arginyl tRNA synthetase
Strain improvement

ABSTRACT

Rifamycins are mainstay agents in treatment of many widespread diseases, but how an improved rifamycin producer can be created is still incompletely understood. Here, we describe a comparative genomic approach to investigate the mutational patterns introduced by the classical mutate-and-screen method in the genome of an improved rifamycin producer. Comparing the genome of the rifamycin B overproducer *Amycolatopsis mediterranei* HP-130 with those of the reference strains *A. mediterranei* S699 and U32, we identified 250 variations, affecting 227 coding sequences (CDS), 109 of which were HP-130-specific since they were absent in both S699 and U32. Mutational and transcriptional patterns indicated a series of genomic manipulations that not only proved the causative effect of *mutB2* (coding for methylmalonyl-CoA mutase large subunit) and *argS2* (coding for arginyl tRNA synthetase) mutations on the overproduction of rifamycin, but also constituted a rational strategy to genetically engineer a reference strain into an overproducer.

1. Introduction

Actinomycetes are prolific producers of a variety of biologically active compounds widely used in medicine as antibiotics, anticancer agents, and immune-suppressant. However, in most cases, wild type strains isolated from nature produce only limited amounts of the desired compounds and thus require genetic manipulations and improvements before they can be used in an industrial setting. Historically, strain improvement is achieved through multiple rounds of random mutagenesis and selection, which, in close association with process optimization, commonly results in high yield, cost-effective large-scale industrial fermentations (Adrio and Demain, 2006).

Since the late 1970s, the accumulation of knowledge on the biosynthetic pathways and genetic control for most of secondary metabolites of commercial interest and the availability of molecular genetic tools paved the way to strain improvement by rational engineering (Baltz, 2001; Vinci and Byng, 1999). Recently, genomics and post-genomics technologies further boosted the strain improvement process through rational, genetic engineering approaches (Blanchard and Hood, 1996; Bro and Nielsen, 2004;

Abbreviations: ABC, ATP-binding cassette; AHBA, 3-amino-5-hydroxybenzoic acid; aminoDAHP, 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate; CDS, coding sequences; DCW, dry cell weight; DEGs, differentially expressed genes; GYM, glucose yeast malt; RP-HPLC-FD, reversed phase high performance liquid chromatography with fluorimetric detection; LAP, Locally Adaptive Procedure; MFS, major facilitator superfamily; NTG, N-methyl-N′-nitro-N-nitrosoguanidine; OMY, oat meal yeast; P_i, inorganic phosphate; PEP, phosphoenolpyruvate; PKS, polyketide synthase; polyP, polyphosphate; ppGpp, guanosine 3′, 5′ bipyrophosphate; PTS, phosphotransferase systems; RMA, Robust Multi-array Average; SDS, sodium dodecyl sulfate; SM, seed medium; SNVs, single nucleotide variations; UV, ultraviolet; PM, Perfect Match; YS, yeast starch

* Corresponding author. Fax: +39 0832 298626.
E-mail address: pietro.alifano@unisalento.it (P. Alifano).

[1] These authors contributed equally to this publication and should be considered co-first authors.

[2] Present address : Molecular Genetics laboratory, Institute for Cancer Research and Treatment, 10060 Candiolo, Torino, Italy.

[3] Present address : Unit of Hematopathology, Department of Hematology and Oncological Sciences "L. and A. Seràgnoli", S. Orsola Malpighi Hospital, University of Bologna Via Massarenti, 9, 40138 Bologna, Italy.

http://dx.doi.org/10.1016/j.ymben.2014.08.001
1096-7176/

Donadio et al., 2007; Lander, 1996; Lee et al., 2005; Stafford and Stephanopoulos, 2001; van der Werf et al., 2005).

Rifamycin B fermentation is a classic antibiotic fermentation that benefited from the traditional mutate-and-screen optimization method over the past 56 years, i.e., since rifamycins were first isolated from a fermentation culture of *Streptomyces mediterranei* (then reclassified as *Nocardia mediterranei*, and finally as *Amycolatopsis mediterranei*) in 1957 at the Lepetit group laboratories (Sensi et al., 1959). Rifamycins are polyketide antibiotics belonging to the family of ansamycins antibiotics, so named because of their basket-like molecular architecture comprising an aromatic moiety bridged at non-adjacent positions by an aliphatic chain. The potent antibacterial activity of these compounds is due to their specific inhibition of bacterial DNA-dependent RNA polymerase (Campbell et al., 2001). Rifamycins are assembled from an aromatic starter unit, 3-amino-5-hydroxybenzoic acid (AHBA), through chain extension by two molecules of malonyl-CoA and eight molecules of (S)-methylmalonyl-CoA to form the initial macrocyclic intermediate proansamycin X. Several tailoring reactions, such as hydroxylation, acetylation, and methylation, are required to form rifamycin S, rifamycin SV, and the final product rifamycin B (Floss and Yu, 2005).

The original strain, *A. mediterranei* ATCC 13685 synthesizes a collection of rifamycins (rifamycin complex), while a single fermentation product, rifamycin B, whose derivatives are effectively used in the clinic, could be obtained in the presence of added diethylbarbituric acid into fermentation media (Margalith and Pagani, 1961a; Margalith and Pagani, 1961b). Subsequently, it was possible to isolate the mutant strain ATCC 21789, capable of producing the sole rifamycin B without any barbiturate addition (Lancini and Cavalleri, 1997), and the related strain S699 (Margalith and Beretta, 1960), widely used in R&D laboratories ever since and, recently, completely sequenced (Verma et al., 2011).

Rifamycin B, the product of the commercial fermentation, has only a very modest antibacterial activity; however, it can be converted chemically, enzymatically, or by biotransformation into rifamycin SV, a biosynthetic precursor of rifamycin B, which has a greater activity and was the first rifamycin used for human therapy (Bergamini and Fowst, 1965). Rifamycin SV can be produced, as a consequence of a block of the terminal conversion step, by the strain U32, a producing organism isolated through mutagenesis (Zhao et al., 2010). However, since the optimization of this mutant has not been as successful as in the case of its parent strains, most commercial fermentations still produce rifamycin B. Rifamycin B is then converted into rifamycin SV and into a large number of semi-synthetic derivatives (including rifampicin, rifabutin and rifapentine) that are widely used in the clinic for treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections (Maggi et al., 1966; Ramos-e-Silva and Rebello, 2001; Sepkowitz et al., 1995).

The biosynthesis of rifamycins in *A. mediterranei* has been widely studied as a model of antibiotic production and a considerable amount of information about the biosynthesis of these compounds and the physiology, genetics, and genomics of the producing strains has been accumulated over the years (Floss and Yu, 2005; Verma et al., 2011; White et al., 1973; Zhao et al., 2010). In particular, several studies allowed the characterization of both the rifamycin biosynthetic cluster (*rif*) in strain S699 and the regulation of rifamycin production and the genetic manipulation of *A. mediterranei* (August et al., 1998; Ding et al., 2003; Lal et al., 1995; Tian et al., 2005; Yang et al., 1998). This evidence undoubtedly contributed to clarify the biochemistry and genetics of rifamycin fermentation and to increase strain productivity. However, the constant demand of rifamycins, and of their semi-synthetic derivatives, which remain mainstay agents in treatment of many widespread diseases in the world, is still hampered by the lack of a rational strategy to improve rifamycin-producing strains.

The present study reports the application of a genomic approach to decipher how the classical mutate-and-screen method led to an improved rifamycin B producer and to define a series of genomic manipulations that constitutes a rational strategy to genetically engineer reference strains into overproducers. This approach takes advantage of low cost genomic sequencing and has recently been applied with success to other antibiotic fermentations leading to the identification of new molecular targets for accelerated strain improvement by genetic engineering (Peano et al., 2012; Rothberg and Leamon, 2008).

2. Materials and methods

2.1. Bacterial strains and media

A. mediterranei S699 was a gift of S. Donadio and M. Sosio (KtedoGen, Milan, Italy). *A. mediterranei* HP-130 is a rifamycin B-overproducing strain that was obtained by the traditional mutate-and-screen method over a period of about 20 years. Available information indicates that *A. mediterranei* ATCC 13685 is the ancestral strain, and that improvement was achieved by 12 sequential rounds of ultraviolet light or N-methyl-N′-nitro-N-nitrosoguanidine (NTG) mutagenesis followed by screening for increased rifamycin B production (Fig. 1A). The strain was also resistant to high rifampicin concentration (250 μg/ml). Strain HP-130 is stored in our laboratory, and it is available to qualified researchers for scientific purpose only.

Strains were stored in 1-ml cryotubes at −80 °C as frozen mycelium in yeast starch (YS) medium containing 15% glycerol at a biomass concentration of approximately 0.25 g dry cell weight (DCW)/ml, or at −20 °C as spores in 20% glycerol (in distilled water) at a titer of approximately 5×10^8/ml. The composition (per liter) of the complete media used in this study is here reported. Seed medium (SM): 4 g peptone, 4 g yeast extract, 2 g KH_2PO_4, 4 g K_2HPO_4, 0.5 g $MgSO_4\cdot7H_2O$, and 10 g glucose (pH 7.2); YS: 2 g yeast extract and 10 g soluble starch (pH 7.3); oat meal yeast (OMY): 40 g oat meal and 1 g yeast extract (pH 6.9); glucose yeast malt (GYM): 4 g glucose, 4 g yeast extract, 10 g malt extract and 2 g $CaCO_3$ (only in solid medium) (pH 7.2); vegetative medium: 10 g defatted soybean meal, 25 g D(+)-glucose monohydrate, 1.5 g propylene glycol, 1 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4\cdot7H_2O$, 1.5 g $CaCO_3$, 1 g $MgSO_4\cdot7H_2O$, 0.01 g $FeSO_4\cdot7H_2O$, 0.0033 g $CuSO_4\cdot5H_2O$, 0.05 g $ZnSO_4\cdot7H_2O$, 0.004 g $MnSO_4\cdot5H_2O$, 0.002 g $CoCl_2\cdot6H_2O$, 0.001 g $(NH_4)_6Mo_7O_{24}\cdot4H_2O$, and 0.5 ml silicon antifoam reagent (pH 7); fermentation medium: 50 g defatted soybean meal, 50 g D(+)-glucose monohydrate, 10 g propylene glycol, 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 5 g $CaCO_3$, 1 g $MgSO_4\cdot7H_2O$, 0.01 g $FeSO_4\cdot7H_2O$, 0.0033 g $CuSO_4\cdot5H_2O$, 0.05 g $ZnSO_4\cdot7H_2O$, 0.004 g $MnSO_4\cdot5H_2O$, 0.002 g $CoCl_2\cdot6H_2O$, 0.001 g $(NH_4)_6Mo_7O_{24}\cdot4H_2O$, and 4 ml silicon antifoam reagent (pH 6.8), when requested media were agarized at a concentration of 1.8%.

2.2. Bioreactor experiments

Vegetative mycelium was used to pre-inoculate a 2 l Erlenmeyer flask containing 200 ml of vegetative medium. Cultures were incubated at 28 °C with shaking at 150 rpm for 72 h; then bacteria were inoculated in fermentation medium at 10% inoculum density, and grown at 28 °C in bioreactors. Bioreactor cultures were carried out on Minifors mini-fermenters (Infors AG, Bottmingen, CH) that operated with a working volume of 1.5 l. Stirring was provided by Rushton-type impellors rotating at 250 rpm. Sterile air was supplied through a sparger. The bioreactors were equipped with pH electrode, pO_2 electrode (polarographic), antifoam probe and Pt-100. Glucose concentration was monitored during fermentation by coupled glucose oxidase–peroxidase reaction using a commercial kit (distributed by Laboser srl). The cell concentration was determined by measuring dry cell weight (DCW). A 10 ml sample composed of culture broth and solid (cells and carbonate) was taken from flask or