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Rifampicin-resistance, *rpoB* polymorphism and RNA polymerase genetic engineering

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ABSTRACT

Following its introduction in 1967, rifampicin has become a mainstay of therapy in the treatment of tuberculosis, leprosy and many other widespread diseases. Its potent antibacterial activity is due to specific inhibition of bacterial RNA polymerase. However, resistance to rifampicin was reported shortly after its introduction in the medical practice. Studies in the model organism *Escherichia coli* helped to define the molecular mechanism of rifampicin-resistance demonstrating that resistance is mostly due to chromosomal mutations in *rpoB* gene encoding the RNA polymerase β chain. These studies also revealed the amazing potential of the molecular genetics to elucidate the structure–function relationships in bacterial RNA polymerase. The scope of this paper is to illustrate how rifampicin-resistance has been recently exploited to better understand the regulatory mechanisms that control bacterial cell physiology and virulence, and how this information has been used to maneuver, on a global scale, gene expression in bacteria of industrial interest. In particular, we reviewed recent literature regarding: (i) the effects of *rpoB* mutations conferring rifampicin-resistance on transcription dynamics, bacterial fitness, physiology, metabolism and virulence; (ii) the occurrence in nature of "mutant-type" or duplicated rifampicin-resistant RNA polymerases; and (iii) the RNA polymerase genetic engineering method for strain improvement and drug discovery.

1. Introduction

Bacterial RNA polymerase (RNAP) is a well established and still an attractive target for antibiotic therapy. RNAP is an essential enzyme with an impressive degree of structural conservation in the three domains of life despite a relatively low sequence identity, and with high degree of both structural and sequence conservation within each domain. These features account for the efficacy, selectivity and broad-spectrum activity of antibiotics targeting bacterial RNAP such as rifamycins (Chopra, 2007; Darst, 2004; Villain-Guillot et al., 2007).

Rifampicin, a semisynthetic rifamycin, is one of the most potent and broad-spectrum antibiotics against bacterial pathogens. The history of this antibiotic (also called rifampin in the United States) dates back to 1957 when a soil sample from a pine arboretum near the beach-side town of St Raphael in southern France was brought for analysis to the Lepetit Pharmaceutical research lab in Milan, Italy. There, a research group isolated an interesting bacterium, currently classified as *Amycolatopsis mediterranei* (Lechevalier et al., 1986), capable of producing a mixture of molecules with antibiotic activity. These related compounds were called "rifomycins" A, B, C, D, E (later changed to "rifamycins") (Aronson, 1999). The only component of this mixture sufficiently stable to be isolated in a pure form was rifamycin B, which unfortunately has only a very modest antibacterial activity. In 1959, after two years of attempts to obtain more stable semisynthetic products, a new molecule with a 4-methyl-1-piperazinaminyl side chain exhibiting high efficacy, good tolerability and excellent oral bioavailability was produced, and was named "rifampicin". Rifampicin and other rifamycins are polyketide antibiotics belonging to the family of ansamycins antibiotics, so named because of their basket-like molecular architecture (Latin: ansa = handle) comprising an aromatic moiety bridged at non-adjacent positions by an aliphatic chain. The potent

Abbreviations: Act, actinorhodin; Crs, catabolite-resistant sporulation; hVISA, heterogeneously vancomycin-intermediate in *S. aureus*; LB, Luria Bertani; MRSA, methicillin-resistant *S. aureus*; NTD, 3,3′-neotrehalosadiamine; PDIM, phthiocerol dimycocerosate; PKS, polyketide synthase; ppGpp, GDP 3′-diphosphate; Red, undecylprodigiosin; REE, rare earth elements; Rif^R, rifampicin-resistant; sVISA, slow vancomycin-intermediate in *S. aureus*; TCA, tricarboxylic acid; TEC, ternary elongation complex; VISA, vancomycin-intermediate in *S. aureus*; VSSA, vancomycin-susceptible in *S. aureus*.

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antibacterial activity of these compounds is due to their specific inhibition of bacterial RNAP (Campbell et al., 2001).

Following its introduction in 1967, rifampicin has become a mainstay of therapy in the treatment of tuberculosis, leprosy and AIDS-associated mycobacterial infections (Shinnick, 1996). In addition to mycobacterial infection, rifampicin is also used in the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in combination with fusidic acid, including difficult-to-treat osteomyelitis and prosthetic joint infections. It is also an excellent prophylactic agent against *Neisseria meningitidis* infections, and is recommended as an alternative treatment for infections with the tick-borne disease pathogens, *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, when treatment with doxycycline is contraindicated. Additional indications are infections sustained by *Listeria* spp., *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Legionella pneumophila*.

As well as for other antibiotics, resistance to rifampicin was reported shortly after its introduction in the medical practice, particularly in tubercle bacilli (Manten and Van Wijngaarden, 1969). Studies in the model organism *Escherichia coli* helped to define the molecular mechanism most frequently involved in rifampicin-resistance (di Mauro et al., 1969; Ezekiel and Hutchins, 1968; Khesin et al., 1969; Tocchini-Valentini et al., 1968). Importantly, the studies with the *E. coli* rifampicin-resistant (Rif^R) mutants also revealed the amazing potential of the molecular genetics to elucidate the structure–function relationships in bacterial RNAP (Jin and Gross, 1991, 1988; Jin et al., 1988a, 1988b; Korzheva et al., 2000; Landick et al., 1990; Mustaev et al., 1997; Tavormina et al., 1996). The scope of this paper is to review how rifampicin-resistance has been exploited to better understand the regulatory mechanisms that control bacterial cell physiology and virulence, and how this information has been used to maneuver, on a global scale, gene expression in bacteria of industrial interest for strain improvement and drug discovery. To this purpose, we start the next section trying to summarize our current understanding about the structure and function of the bacterial RNAP, the interaction of RNAP with transcription factors and rifampicin, and the so-called "stringent response", the global regulatory system that modulates gene expression in bacteria in response to nutritional stress. Indeed, a functional overlap has been reported between the phenotypes exhibited by certain rifampicin-resistance mutations and the stringent phenotype (Xu et al., 2002; Zhou and Jin, 1998).

2. The bacterial RNA polymerase, the interaction with rifampicin, and the "stringent response"

2.1. The bacterial RNA polymerase

Over the past twenty years, crystallographic structures have been determined for bacterial RNAP complexes with nucleic acids, nucleotides, modulating small molecules, and inhibitors including rifamycins. The structure of *Thermus aquaticus* core RNAP, solved in 1999, revealed that the enzyme with a subunit composition of $\alpha 2\beta\beta'\omega$ (Murakami et al., 2002a,b; Vassylyev et al., 2002; Zhang et al., 1999) has a shape reminiscent of a "crab claw", which is similar to that of the archaeal RNAP (Hirata et al., 2008) and eukaryotic RNAP (Cramer et al., 2000, 2001). The two largest β and β′ subunits form the two pincers of the crab claw, which are separated by a deep cleft about 27 Å wide (Zhang et al., 1999) (Fig. 1A). The two pincers of the claw form a positively charged cleft (known as the active-site or main channel) which contains two catalytically active Mg^{+2} ions, and accommodates the nucleic acids during transcription. A dimer of α subunits (αI and αII) is positioned at the interface of the β and β' pincers, while the small ω subunit wraps around the C-terminal tail of the β' subunit. Each of the two α subunits consists of two domains: an N-terminal domain (α-NTD) and a smaller C-terminal domain (α-CTD) joined by a flexible linker (Blatter et al., 1994). The α-NTD dimerizes and is responsible for the assembly of the large β and β' subunits. The α-CTD plays a different role; it interacts with a diverse range of transcriptional activators and can interact with promoter DNA sequences (Gourse et al., 2000; Haugen et al., 2008).

Based on crystallographic data, biophysical results, biochemical results, models have been proposed for the structures of transcription initiation and elongation complexes. The models propose that nucleic acids completely fill the active-site (or main) channel of RNAP, such that the only route by which incoming nucleoside triphosphate substrates (NTPs) can access the active center is through a narrow funnel-shaped "pore" (also known as the "secondary channel") that leads from the surface of the enzyme to the active site (Gnatt et al., 2001; Ebright, 2000). Crystallographic data show that α-NTD, ω and regions of β and β′ subunits form an immobile core surrounded by five domains: the β′ clamp, β′ jaw, β-flap, β protrusion (or β upstream lobe) and β lobe (or β downstream lobe) (Fig. 1A). These domains are able to move independently from each other as rigid bodies, and their movements enable binding to σ factors and promoter DNA, and opening and closing of the main channel during the transcription cycle, driving loading of the DNA into the active site channel. In particular, the β′ clamp folds over the catalytic cleft and holds the nucleic acids (DNA and RNA) more tightly in the active center, stabilizing the elongation complex. Movements of the β upstream and β downstream lobes contribute to open and close the active site channel, while conformational changes of the β flap modulate the elongation behavior of the enzyme at the RNA-exit channel in response to hairpin-dependent pause signals.

In addition to the above-mentioned mobile domains, crystallographic data have also shown other structural motifs (helices or loops) that have been named according to either location or aspect, or presumed role in the transcription process as deduced also by genetic, biochemical and biophysical studies (Fig. 1A). For examples, the β′ bridge helix separates the main channel into a downstream DNA entry channel and secondary channel, and is located near the template DNA at the +1 site. Therefore, it has been proposed that this motif is involved in translocation of the nucleic acids during transcription. Just inside the secondary channel lies the β′ trigger loop, a structural element that was shown to play a role in elongation by modulating the oscillating properties of the adjacent β′ bridge helix. The location of the β′ rudder, the β′ lid and the β fork loop 1 suggests that these loops are involved in DNA-RNA strand separation, in order to maintain as 8–9 bp long DNA-RNA hybrid of the transcription "bubble" in the active-site cleft. The fork loop 2 and the zipper could be involved in delineating the downstream and upstream boundary of the transcription bubble, respectively. Five loops of β′ and β subunits have been termed the switches and could participate in controlling the position of the clamp or, for switches 1–3, in forming a binding site for the DNA–RNA hybrid.

2.2. Interaction of RNA polymerase with sigma factors and regulatory proteins

Although core RNAP is catalytically proficient for transcription, it can only weakly and unselectively bind to the DNA. To begin transcription at a specific promoter, RNAP must first bind a dissociable subunit called the sigma (σ) factor to form a fully functional RNAP holoenzyme (referred to as Eσ). The multiple members of the σ factor family are divided into two classes, the σ^{70} class and the σ^{54} class, with little sequence conservation between the two. The σ^{70} class owes its name to the prototypical *E. coli* "housekeeping" σ^{70} factor. This class is composed of the primary σ factors, which are responsible for transcribing most genes involved in basic cellular metabolism, and many members of alternative σ factors, which

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