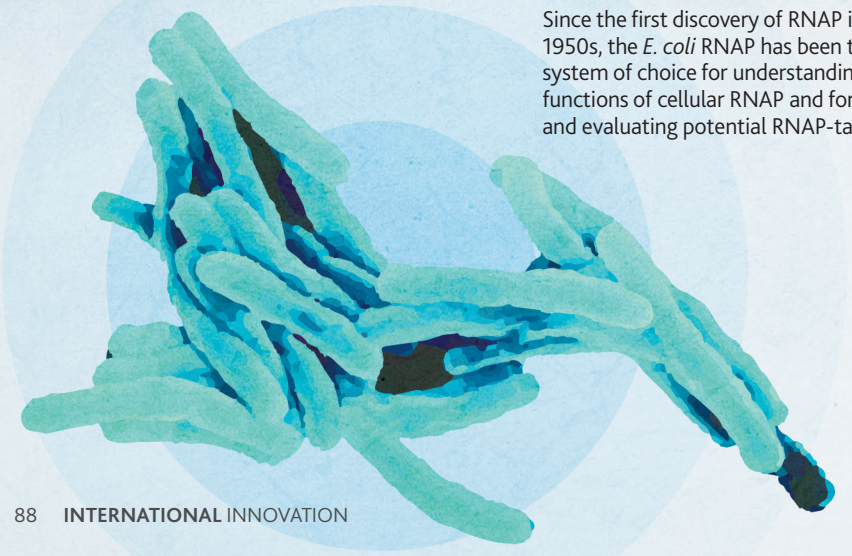
Targeting multidrug resistance in tuberculosis treatment

DR KATSUHIKO MURAKAMI

The study of how genetic code is expressed in molecular terms is critical to understanding and tackling diseases, according to **Dr Katsuhiko Murakami**. Here, he describes his collaborative efforts to develop new antibiotics using X-ray crystallography and what first sparked his interest in this area

What are the objectives of your study of bacterial RNA polymerase (RNAP)?

RNAP is a central player for expressing genes and is able to carry out very complicated enzymatic reactions with high speed and accuracy. The key to understanding how this amazing enzyme works is to observe its three-dimensional structures in the different stages of reaction. Among the many methods available for structural studies of biological macromolecules, I have chosen X-ray crystallography, since it can image large macromolecule structures, like RNAP, at high resolution. While seeing the structure sometimes enables me to answer questions, it usually prompts more questions! I will keep chasing new structures of this enzyme in the different stages of reaction until the end of my career.



Can you identify what first drew you to this area of research, and are there any individuals you consider as personal heroes within the field?

I have a very high regard for my PhD supervisor, Professor Akira Ishihama, who gave me an opportunity to work in his laboratory despite having no knowledge of RNAP nor any previous training in biochemistry or molecular biology. He taught me the basics of RNAP purification and characterisation. This foundation helped me greatly later on when making high quality crystals, since good protein preparation is key for success in X-ray crystallography. Professor Ishihama also delivered very informative lectures on RNAP research history, which originally inspired my idea of using basic research into the interaction between RNAP and rifampicin for clinical application.

For over four decades, rifampicin has been used as a first line antibiotic treatment of tuberculosis (TB). How effective is this drug?

Rifamycin and its semi-synthetic derivatives, including rifampicin, were a tremendous breakthrough when developed in the 1960s, particularly when they were shown – in combination therapy with other TB drugs – to reduce the overall TB treatment time from 18 to nine months.

How does the determination of the crystal structure of the *E. coli* RNAP aid antibiotic development?

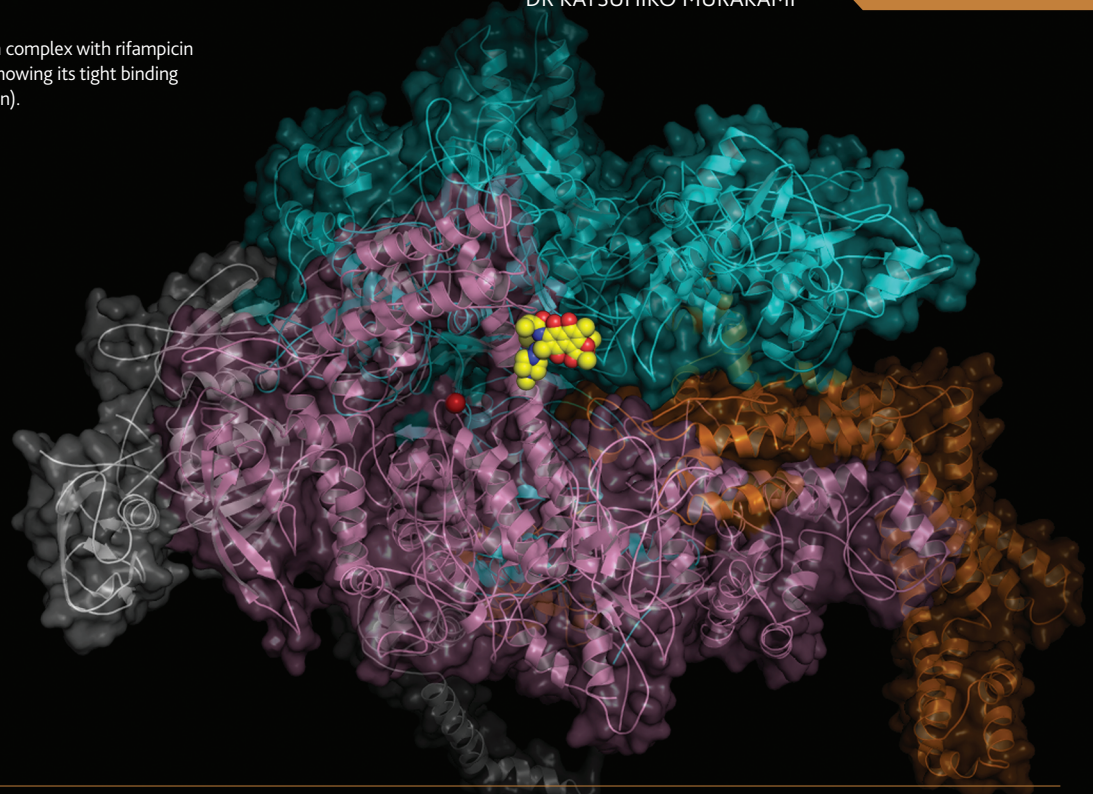
Since the first discovery of RNAP in the early 1950s, the *E. coli* RNAP has been the model system of choice for understanding the functions of cellular RNAP and for screening and evaluating potential RNAP-targeting antibiotics, including rifampicin. In addition, since the sequence and antibiotic sensitivity of *E. coli* RNAP are similar to those of pathogen-related RNAPs, including *Mycobacterium tuberculosis* and *Staphylococcus aureus*, *E. coli* polymerase can now be used to readily study RNAP-antibiotic interactions by X-ray crystallography. Other important advantages of working with *E. coli* RNAP are that it can be prepared from a protein overexpression system, and recombinant RNAP makes for good crystal quality, so we can solve structures of mutants such as rifampicin-resistant mutants.

At what stage is this work currently?

Although many rifampicin-resistant strains of TB can be made in cell cultures, in clinical isolates, only three specific mutants account for nearly 85 per cent of rifampicin-resistant TB strains. We have solved the crystal structures of these three rifampicin-resistant mutants of RNAPs to study how rifampicin-resistant RNAP changes the shape of the rifampicin-binding pocket to reduce the affinity of rifampicin. From these structures, we have been designing rifampicin derivatives *in silico* for fitting compound nicely to each of the altered rifampicin-binding pockets to inhibit mutant RNAP activity. My collaborators, Professors George Garcia and Hollis Showalter, then synthesise new rifampicin derivatives and test their activities against them. Our goal is to develop three new compounds for each of three mutants that would save 85 per cent of patients suffering from rifampicin-resistant TB.

Finally, what backing have you received to date and what opportunities have you been afforded by this support?

My laboratory has been funded by the National Institutes of Health. In addition, I had support from the Pew Scholars' Programme in the Biomedical Sciences. The Pew programme gave financial support to a risky research project and the programme meetings also provided the opportunity for me to interact with many prominent scientists that opened my eyes to think about how to contribute to biomedical research via basic research in academia. Finally, I have been supported by my family, my wife Shoko and three sons Taiki, Yuta and Hiroya. They enrich my life, and that allows me to focus on research in the laboratory.



X-ray crystal structure of the *E. coli* RNAP in complex with rifampicin (yellow/blue/red cpk model in the centre) showing its tight binding pocket found in the RNAP beta subunit (cyan).

Pocket of hope

Analysis of X-ray crystal structures of bacterial and archaeal RNA polymerases at **Pennsylvania State University** is not only revealing the basic mechanisms and pathways of genetic information transcription into RNA in all life forms, but is paving the way for developing new antibiotics against drug-resistant forms of tuberculosis

EVIDENCE OF TUBERCULOSIS (TB) infection in prehistoric human remains indicates that the disease has a very long history, though it was only in the 17th Century that it was identified as damaging the lungs and not until the 19th Century that its capacity for air-borne transmission between people became apparent. Without a known cause, public health management of consumption, as it was known, consisted of isolation of those infected in remote sanatoria; but once Robert Koch showed in 1882 that TB was caused by *Mycobacterium tuberculosis*, and it was discovered that unpasteurised milk was a source of original infection, the Bacillus Calmette-Guérin (BCG) vaccine was soon developed and the disease fell under a degree of control. Nevertheless, it is estimated that 100 million people died from TB in the 20th Century and the toll would have been higher if the streptomycin antibiotic had not been developed in the mid-1940s.

In 1966, a new antibiotic drug was developed for first line treatment of TB, and was found to have a high degree of success. This was rifampicin (also known as rifampin), a semi-synthetic derivative of rifamycin and an inhibitor of bacterial RNA polymerase (RNAP). However, adherence to treatment with rifampicin among patients has traditionally been poor, as the drug can take up to nine months to work; this and reliance on a limited range of treatment options for TB for more than 40 years has resulted in the emergence of multidrug-resistant strains of TB. Among the 8 million new cases reported each year, more than 300,000 involve multidrug-resistant strains of pulmonary TB. This places a large burden on both patients and health services: second line treatment for these resistant strains entails two years of chemotherapy.

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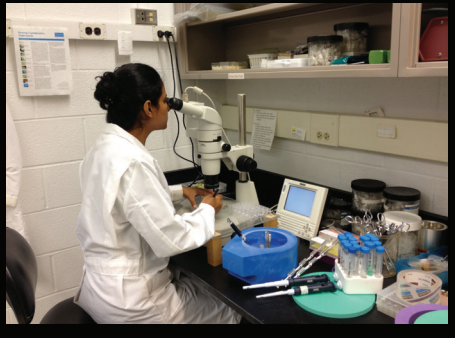
Although in decline worldwide, numbers of TB cases are now on the rise in cities of developing countries. As recently as 2011, 1.4 million people across the world died from the disease: it is the second most common cause of death among people from infection by a single agent, after HIV/AIDS. A complicating factor is that TB is more likely to affect those whose immune system is compromised and it also exacerbates their condition; it is estimated to cause a quarter of the deaths among HIV sufferers.

Dr Katsuhiko Murakami is a molecular biologist and biochemist at Pennsylvania State University whose research interests centre on the structural and mechanistic enzymology of prokaryotic RNAPs. Because RNAP enzymes are highly conserved between prokaryotes (including archaea and bacteria) and eukaryotes, Murakami's work with RNAP in prokaryotes enables structural comparisons and provide the basic transcription mechanisms in all life forms, and he hopes to provide useful structural frameworks for elucidating the transcription mechanisms. In addition, Murakami is currently working on a project in collaboration with Professors George Garcia and Hollis Showalter from the University of Michigan to develop new antibiotics for treating TB that will be effective against multidrug-resistant strains. In the project, the three laboratories are employing a combination of X-ray crystallography, *in silico* modelling, synthetic organic chemistry and high-throughput screening approaches to target the pathogen's RNAP.

RNAP AND RIFAMPICIN

RNAP is an enzyme essential to all life forms, producing RNA from DNA. Rifampicin works by



Ritwika Basu, a graduate student in Murakami's lab, freezing RNAP crystals for X-ray crystallographic data collection. Protein crystals are usually smaller than 0.2 mm thereby requiring microscopy to catch them.

X-RAY CRYSTALLOGRAPHIC STUDIES OF NUCLEIC ACID POLYMERASES

OBJECTIVES

To determine the X-ray crystal structures of prokaryotic RNA polymerases including bacterial, archaeal and bacteriophage and their complexes with auxiliary protein factors, nucleic acid and inhibitors.

KEY COLLABORATORS

Professor George Garcia; **Professor Hollis Showalter**, University of Michigan

Dr Lucia Rothman-Denes, University of Chicago

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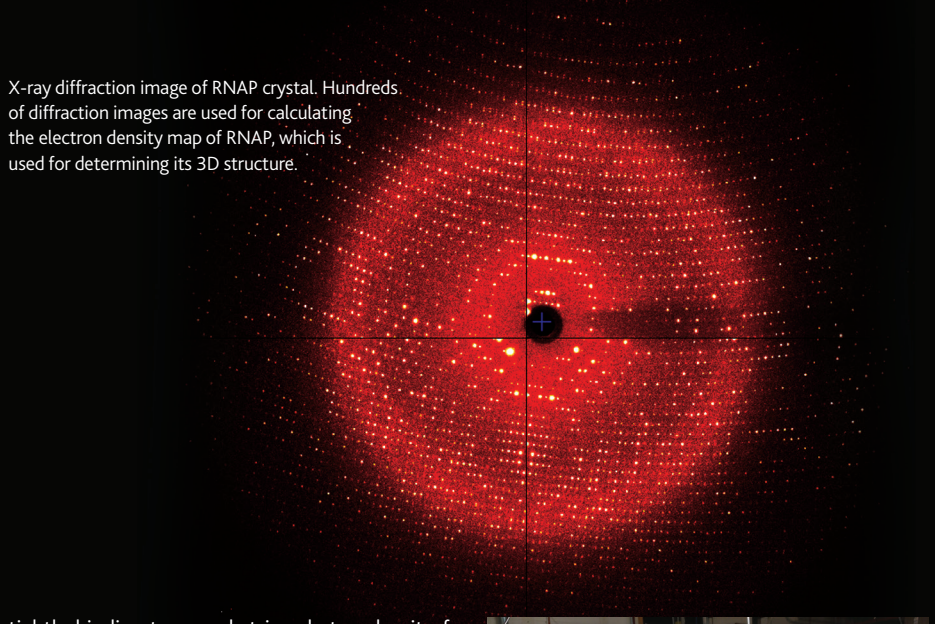
KATSUHIKO MURAKAMI received his PhD from the Graduate University of Advanced Studies in Japan in 1997. He engaged in postdoctoral study at the Rockefeller University, then joined the Department of Biochemistry and Molecular Biology at Pennsylvania State University as an Assistant Professor in 2003 and became an Associate Professor in 2009.



X-ray diffraction image of RNAP crystal. Hundreds of diffraction images are used for calculating the electron density map of RNAP, which is used for determining its 3D structure.

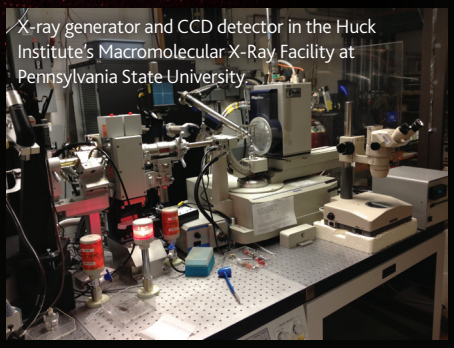
tightly binding to a pocket in a beta subunit of the bacterial RNAP to inhibit RNA transcription and so render the bacterium unviable. Using *Escherichia coli* as a model system, Murakami's approach has been to determine the crystal structure of RNAP to pinpoint the binding modes for rifampicin in mutant forms, therby guiding the design of new rifampicin derivatives.

Murakami prepares *E. coli* RNAPs from a protein overexpression system and then determines their structures by X-ray crystallography. In this way, he has been able to determine the crystal structures of the RNAPs of the major forms of rifampicin-resistant mutants: "Although many rifampicin-resistant strains of TB with binding pocket mutations have been isolated in bacterial culture, there are only three specific mutations that account for most cases of rifampicin-resistant TB," he explains. Murakami has found that each rifampicin-resistant RNAP structure displays a unique conformation of the rifampicin binding pocket; their structural deviations from the normal rifampicin binding pocket in non-resistant *E. coli* RNAP are also consistent with degree of resistance, suggesting that rifampicin resistance basically derives from the complementarity of the alternative shape of the binding pocket and the structure of rifampicin.

PROMISING COMPOUNDS FOR NEW ANTIBIOTICS

From the many possible synthetic rifamycin derivatives available, Murakami, Garcia and Showalter have selected the benzoxazinorifamycins, including Rifalazil, which display superior affinity toward both normal and rifamycin-resistant mutant forms of RNAP. These compounds also have little effect on hepatic cytochrome P450 enzyme induction, so will not lead to complications induced by adverse drug interactions if used in combination therapy for HIV. Owing to these properties, Murakami considers that the benzoxazinorifamycins have great potential for TB treatment.

Murakami has determined the crystal structures of the *E. coli* RNAP complexes with two of the benzoxazinorifamycins. From this, he has found that the ansa/naphthalene sectors of the



X-ray generator and CCD detector in the Huck Institute's Macromolecular X-Ray Facility at Pennsylvania State University.

benzoxazinorifamycins molecules bind in a deep pocket of the beta subunit of the RNAP, so blocking the path and progression of RNA transcription. Also, the tail of one benzoxazinorifamycin fits into a cavity between the beta subunit and the sigma factor that initiates RNA transcription in bacteria, so Murakami and his collaborators propose that this extra interaction also influences the template DNA position, reducing the efficiency with which transcription starts: "A challenge here is that one of the rifampicin-resistant mutants changes its shape a great deal, so we have to change the rifampicin structure substantially to fit into the binding pocket. Unfortunately, since rifampicin is a semi-synthetic compound from rifamycin, the numbers of chemical groups that can be modified is limited," Murakami observes.

The researchers intend to continue exploring the structure-activity relationships of the benzoxazinorifamycins and their role in RNAP inhibition, toward identifying superior chemical compounds with substantial potential for development as antibiotics for TB: "New antibiotic development takes more than 10 years and requires a lot of work in many different research areas, that is why of course I cannot manage the entire process by myself. Indeed, I can contribute only a small part, which is solving the X-ray crystal structure of the RNAP and inhibitor complex, but I do believe that I can provide higher quality structures than anyone else," reflects Murakami. "I hope that the crystal structure from my laboratory will provide a good framework for understanding how the inhibitor binds RNAP, to help the design of better antibiotics for TB treatment and also speed up their development."