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Molecular Biology of Bacteria

Rajarshi Kumar Gaur
Hemant K. Gautam
Editors

Microbiology Research Advances

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MOLECULAR BIOLOGY OF BACTERIA

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Preface

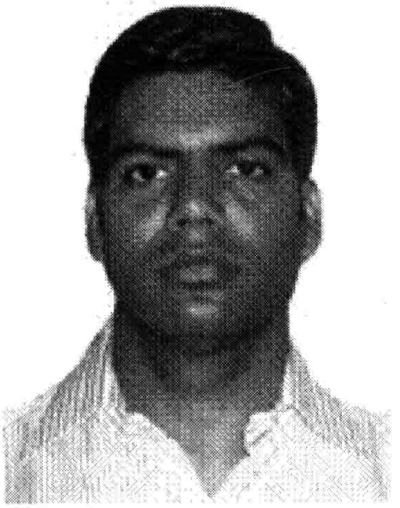
Bacteria are not just important as laboratory tools to understand higher organisms; they are important and interesting in their own right. For instances, they play an essential role in the ecology of earth. They are the only organisms that can fix atmospheric nitrogen, that is convert N_2 to ammonia, which can be used to make nitrogen-containing cellular constituents such as proteins and nucleic acids. Moreover bacteria produce most of the naturally occurring so-called greenhouse gases, such as methane and carbon dioxide, which are in turn used by other types of bacteria.

Bacteria are also worth studying because of their role in disease. They cause many human, plant and animal diseases and new disease are continuously appearing. Knowledge gained from the molecular genetics of bacteria will help in the development of new ways to treat or otherwise control old diseases, as well as new ones.

Molecular Biology has proved to be one of the more fruitful technological approaches sciences, being both very powerful and able to generate valuable intellectual property. This book aims to present examples in the application molecular biology and genetic engineering in bacteriology. This book shows diverse roles of bacteria in the ecosystems and it gives significant contributions from biotechnology approaches. The challenges of these aspects and methodologies inspired us to edit the book entitled *Molecular Biology of Bacteria.*

Rajarshi Kumar Gaur
and
Hemant K. Gautam
Editors

About the Editors



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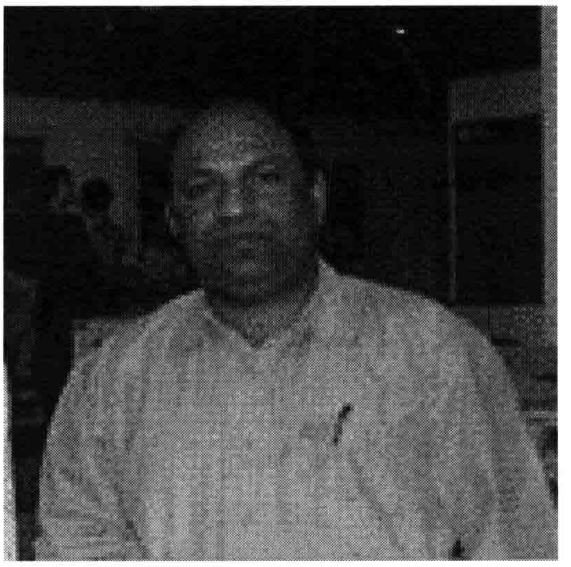

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Contents

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Chapter 1

Intergeneric Conjugation: A Practical Method for Genetic Manipulation in Actinomycetes

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Abstract

The system for intergeneric conjugation has been developed for many actinomycete species including *Streptomyces* and rare actinomycetes. *E. coli* donor strains generally used are the methylation proficient strain, S17-1 harbouring *tra* genes on its chromosome and the methylation-deficient strain, ET12567 containing *tra* genes in a self-transmissible plasmid pUB307 or a non-transmissible plasmid pUZ8002. Integrative plasmids used are normally non-replicative plasmids carrying the *oriT* for the propose of gene disruption (e.g. pSET151, pIJ8671) or with additional ϕC31 *attP-int* system in the case of gene expression (e.g. pSET152, pIJ8600). Actinomycete recipients used for mating with *E. coli* could be in spore suspension with or without pre-germination process, or mycelia in liquid culture. Several agar media including MS and ISP 4 were generally used for

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streptomycete strains while other more specific media were proposed for rare actinomycetes. Concentration of $MgCl_2$, ratio of donor and recipient and other parameters are significantly important to increase conjugation efficiency. The intergeneric conjugation approach has become a powerful method to genetically manipulate actinomycete species with high efficiency. It turns out to be a common tool to characterise functions of genes/gene clusters in these pharmaceutically important bacteria.

Introduction

Actinomycetes are high G+C content (74 mol%) filamentous Gram-positive bacteria found abundantly in soil, sand and sediments. They have a complex life cycle with an extended substrate mycelia on which aerial hyphae is mostly formed with exospores. Genus *Streptomyces* in particular is a key producer of valuable secondary metabolites including anti-bacterials, anti-fungals, anti-cancers, immunosuppressants, herbicides and insecticides used nowadays in medicine, pharmaceutics and agriculture [1, 2]. Actinomycetes, therefore, are the most attractive microorganisms to study genes involved in biosynthesis of such important secondary metabolites. Recently, genome analysis of *Streptomyces* chromosomes revealed that they carry vast numbers of uncharacterised genes and biosynthetic gene clusters for undiscovered secondary metabolites [3]. Therefore, the establishment of efficient genetic manipulation systems to introduce homologous/heterologous DNAs into actinomycete species has become a critical requirement for achieving functional analysis of the genes/gene clusters of interest.

Genetic manipulation in actinomycetes was developed intensively for the genus *Streptomyces*. Recombinant plasmids construction for gene expression and disruption/replacement can be generally transferred into streptomycete hosts using polyethylene glycol (PEG) mediated protoplast [4]. The procedure is based on the protocol defined for *S. coelicolor* and *S. lividans* [5]. However, this method is time-consuming procedure as it requires high productivity of protoplast formation and regeneration to achieve good transformation efficiency. With a number of different streptomycete species, the method has to be optimised e.g. mycelial age, concentration of the lysozyme to give good protoplast formation and conditions for protoplast regeneration. PEG mediated protoplast transformation does not easily applicable to every species and frequently results in low efficiency particularly with rare actinomycete strains. In addition, due to the strong host restriction modification system of *Streptomyces* [6], it is essential to find a proficient method to transfer DNA with high efficiency. Although high efficient transformation by electroporation was proposed to avoid the problematic protoplast formation and regeneration [7, 8], it requires luxurious equipment and has limited application not to every species of actinomycetes.

Nowadays, the method generally employed for genetic manipulation in *Streptomyces* and other genera is plasmid-mediated intergeneric conjugation with *E. coli* as a donor [9]. This method is proved to be simple and less time consuming and likely to be applied to various actinomycete species. However, the efficiency of this system varies significantly from one species to another, and it is necessary to improve experimental procedures for a new species.

Bacterial Conjugation

Conjugation is a common phenomenon in both Gram-negative and Gram-positive bacteria. It requires close physical contact between donor and recipient cells and is usually mediated by plasmid-encoded proteins which provide transfer (*tra*) functions [10, 11, 12].

The conjugation by self-transmissible plasmid is related in a mechanism sense to that of F plasmid in *E. coli*. Donor creates F pilus to make cell-to-cell contact to recipient. The mechanism is initiated by creating a single-stranded nick at the origin of transfer (*oriT*). The 5'-end of the nicked DNA is transported linearly through the F pilus into the recipient cell where it is replicated; while, the unnicked DNA is replicated in the donor cells [13].

Naturally, some self-transmissible plasmids could transfer among the same bacterial species but not to other different species. However, some plasmids were able both to transfer and to replicate in effectively different species. Some conjugation phenomena with non-pilus mechanisms were also found in Gram-positive bacteria including *Streptomyces* [10].

Conjugation within *Streptomyces* Species

The conjugation process with non-pilus mechanism naturally appears in *Streptomyces*. It is a very distinct process from other bacteria in its molecular mechanism and its visible phenotypic appearance. It occurs through intermycelial transfer from the hyphal tips of a plasmid carrying donor to recipient [14, 15]. It seems that such single-stranded DNA transfer that appears in all conjugation systems in unicellular bacteria does not involve in *Streptomyces* conjugation.

Conjugative plasmid was transferred as a double-stranded DNA molecule [15, 16] by *tra* gene products, e.g. *traB* for pSN22 [17] and *traSA* for pSAM2 [16] during mating [18]. Then the plasmid is intramycelial spread through a septum to the next cell of the recipient mycelium. At this point, the growth is retarded in order to obtain sufficient copy number of the plasmids for the next spreading to another compartment of the mycelium [14, 19]. This delay causes a pock formation, a visible retardation zone of plasmid-acquiring recipient cells grown in a confluent lawn of plasmid-free recipients [20, 21].

Intergeneric Conjugation between *E. coli* and *Streptomyces*

Conjugal transfer from *E. coli* to Gram-positive bacteria with F plasmid-like system was demonstrated by Trieu-Cuot et al. [22]. In 1989, intergeneric conjugation from *E. coli* to *Streptomyces* was firstly described [9] and later has been developed and applied to several genera of *Actinomycetales*. This method has proved to be easy, convenient and less time-consuming compared to the original PEG-mediated protoplast transformation method.

The system of intergeneric conjugation between these two taxonomically different microorganisms is absolutely required an *oriT* and *tra* genes for transfer functions in the donor *E. coli* host. It is hypothetically explained that a plasmid was transferred from *E. coli* to *Streptomyces* by single-stranded DNA forms generated by *oriT*-endonuclease function of

donor [10]. This transfer method then could overcome problems of the restriction barrier to foreign DNA in *Streptomyces* hosts by the transfer of single-stranded DNA [23].

E. coli Donor and Mobilisable Plasmids

E. coli donor strains generally used for intergeneric conjugation are the methylation proficient strain, S17-1 harbouring RP4 *tra* genes on its chromosome [9], the methylation-deficient strain, ET12567 (*dam-13::Tn9 dcm-6 hsdM*) [24] containing *tra* genes in a self-transmissible plasmid pUB307 [23] and ET12567 carrying a non-transmissible plasmid pUZ8002 [25]. pUZ8002, a helper plasmid RK2 derivative, is not efficiently transferred itself because of a mutation in its own *oriT* but can supply transfer functions to mobilise *oriT*-carrying non-conjugative replicon plasmids [26]. By using methylation deficient donor such *E. coli* ET12567, the restriction barriers of foreign DNA could be reduced and efficiency of DNA introduction could be highly increased in *Streptomyces* host [6, 23].

The plasmids used for intergeneric conjugal transfer are mobilisable plasmids that required *tra* genes function of F plasmid located on either host chromosome (e.g. *E. coli* S17-1) or helper plasmid (e.g. pUZ8002). There is no evidence yet whether a pilus mechanism is required for mating pair formation between *E. coli* and *Streptomyces*.

Non-replicative plasmids are used on propose of integration of genetic materials into *Streptomyces* chromosomes. These plasmids carry the origin of replication for *E. coli* and the *oriT* region of the broad host range conjugative plasmid RK2 [9, 10]. If the plasmids required for gene expression propose, integrase gene (*int*) and attachment site (*attP*) of *Streptomyces* phage ϕC31 were inserted in order to drive site-specific recombination at *attB* site of *Streptomyces* chromosome [27, 28]. These plasmids, for example, are pIJ8600 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r, Thio^r, *tipA*p) [29], pSET152 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r) [28], pIJ6902 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r, Thio^r, *tipA*p) [30] and pIJ10257 (*ori*pUC, *oriT*, ϕBT1 *attB-int*, Hyg^r, *ermE*p*) [31]. If the plasmids are to use for integration by homologous recombination, no *int* and *attP* of ϕC31 was required e.g. pIJ8671 (*ori*pUC, *oriT*, Apr^r, Thio^r, *tipA*p) [29] and pSET151 (*ori*pUC, *oriT*, Thio^r) [32].

The non-replicative recombinant plasmid is forced to integrate at the target homologous gene to achieve disruption. Some shuttle plasmids were designed for replication purpose in *Streptomyces* then the origin of replication for both *E. coli* and *Streptomyces* was inserted such as pJN100 (*ori*pUC, *ori*pIJ101, *oriT*, Apr^r, *snpA*p) [33].

Pre-Treatments for Streptomycete-Actinomycete Recipients

Pre-germination of spores of *Streptomyces* recipients by heat treatment is critical for intergeneric conjugation procedure [5, 23]. The temperature and time used for spore pre-germination treatment effect on the efficiency of conjugation in individual species. In general, pre-germination of *Streptomyces* spores activated by heat treatment at 50°C for 10 min before mating with an *E. coli* donor was recommended [5, 9, 23]. The spore pre-germination of *S. lividans* at this condition increased the efficiency of conjugation by 5-10 times [9]. It was practically used for spores of *S. albus*, *S. anibioticus*, *S. aureofaciens*, *S. bambergiensis*, *S.*