

**ISOLATION AND MOLECULAR  
CHARACTERIZATION OF PROMOTERS FROM  
*GORDONIA***

**JANANEE JAISHANKAR**



**DEPARTMENT OF BIOCHEMICAL ENGINEERING  
AND BIOTECHNOLOGY  
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**JUNE 2021**

© Indian Institute of Technology Delhi (IITD), New Delhi, 2021

**ISOLATION AND MOLECULAR  
CHARACTERIZATION OF PROMOTERS FROM  
*GORDONIA***

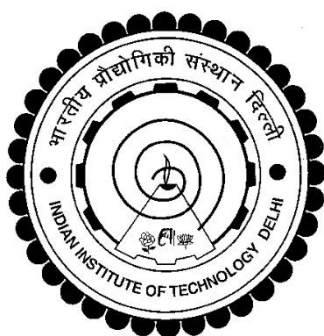
by

**JANANEE JAISHANKAR**

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND  
BIOTECHNOLOGY

Submitted

In fulfilment of the requirements of the degree of Doctor of Philosophy  
to the



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**JUNE 2021**

## CERTIFICATE

This is to certify that the thesis titled “**Isolation and molecular characterization of promoters from *Gordonia***” being submitted by **Ms. Jananee Jaishankar** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** is a record of bonafide research work carried out by her under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi.

The results presented in this thesis have not been submitted in part or full to any other University or Institute for the award of any other degree or diploma.

Date

Dr. Preeti Srivastava  
Associate Professor  
Department of Biochemical  
Engineering and Biotechnology  
Indian Institute of Technology  
Delhi  
New Delhi- 110016

## ACKNOWLEDGEMENTS

There have been innumerable people who have helped me directly and indirectly towards my Ph.D. I would like to acknowledge everyone for their significant contribution in shaping my research career and helping me navigate tough times.

I am highly grateful to my supervisor, Prof. Preeti Srivastava for helping me grow both professionally and personally. I greatly admire her passion for science and her hard-working nature which have been one of the biggest motivations throughout my Ph.D. Words cannot express how her encouragement has given me the much-needed push when things were not going smoothly, while she also patiently answered my doubts. She has also provided me with several exciting opportunities to present my work. I will always remember the interesting discussions we had during our meetings and the sheer joy on her face whenever a paper got accepted or I received an award. I hope to carry forward the enthusiasm she has for research and teaching in my future endeavours.

I would like to express gratitude to my research committee members: Prof. Saroj Mishra, Prof. Biswajit Kundu and Prof. Shilpi Sharma for evaluating my work from time-to-time and giving valuable suggestions for improving my work. I would also like to thank Prof. D. Sundar, Head of Department, and other faculty members of DBEB for teaching interesting courses and offering support during the last five years.

I am extremely thankful to everyone of RNA-I lab for their help and assistance during the last five years. My seniors, Dr. Pooja Murarka and Dr. Divya Singhi have been immensely supportive both in managing the lab environment and for their suggestions related to work. I am also grateful to my good friend Rohit, for his continuous motivation and for cheering me up when things did not work out. Also, thanking him for all the times he has collected my samples during late night hours. I would definitely miss all the lunch and coffee time conversations with Rohit, Divya and Pooja ma'am.

I would further like to show my appreciation to the current members of RNA-I lab: Arif for being a great friend and good lab partner, Aditi for being such a lively person and for her help with Western Blotting, Deeksha for all the gossips and phone conversations about random things, Krishnapriya for her continuous encouragement, Shabnam for her understanding nature, Jyoti for her motivation, and Priyanka and Sourabh for their support.

I am also thankful to my mentees Jayaram, Prarthana and Abhishek; spending time discussing their work has given me new ideas for my own work. It was fun working with them. I would also like to acknowledge my other mentees who supported me in the lab. Thanks are also due to Ayushi, Bintu, Mansi, and other BTPs and MTPs for maintaining a lively environment in the lab.

Extending my gratitude to Dr. Pooja Singh, Ankur sir and Sabita for their help during the initial days in the lab.

Special thanks to Amit bhaiya for maintaining a clean lab environment and for his assistance in processing of bills.

I am also grateful to the members of DBEB and CBME labs for allowing me to use specific instruments. I would also like to thank Upma for her help in qPCR, Shashi for help with the bioreactor, and Srishti for her suggestions related to Western Blotting.

I cherish the time I have spent with amazing batchmates including Nudrat, Manju, Srishti, Rabab, Shashi, Rishabh, Arun, Navodit and Rohit, meeting in the hallway and supporting each other throughout has definitely been helpful.

I would also like to thank my husband Ashwin for being so supportive during the final stages of my thesis. His constant encouragement and advice helped me complete my doctorate successfully. I truly appreciate my in-laws for helping me stay calm and am thankful to them for their continuous encouragement and motivation.

I am highly grateful to the people without whom nothing in my life would have ever been possible: Mom, Dad and my extremely supportive sister Nandhini. I cannot express in words how much they mean to me, the way in which they always motivate me, listen to my never-ending rants, help me stay positive and persevere. My accomplishments are only because of their never-ending love, care and blessings. I am fortunate for their continuous belief in me which has helped me in the pursuit of excellence. Many many thanks to Nandhini, for taking most of the family responsibility on her shoulders and giving me a dose of positivity every time I require. I dedicate this thesis to them.

Special thanks to Paati (my grandmother), a person who has always believed I will do something great in life. I hope you are watching me happily from heaven and showering your blessings for my thesis submission.

Finally, I would like to express my humble gratitude to God, for always being my strength and guiding light.

**Jananee Jaishankar**

## ABSTRACT

Novel regulatable promoters serve as effective tools for the construction of gene expression systems. In this aspect, promoters with varying strength and regulatability are required for use in different process-specific applications. Understanding the promoter functionality and characteristics is therefore essential for their exploitation. In this study, a previously isolated stationary phase promoter from *Gordonia* sp. IITR100 was engineered by repeated rounds of random mutagenesis to obtain a strong synthetic promoter, that offers stationary phase inducibility and higher strength as compared to the wild-type promoter in *E. coli*.

To isolate additional promoters from *Gordonia* sp. IITR100, the whole-genome sequence was determined, facilitating the identification of core promoter sequences. Further, the consensus sequence of the core promoter regions was deduced, which revealed the presence of conserved extended -10 region. Such a feature has also been reported in other members of Actinobacteria including *Corynebacterium* spp., *Rhodococcus* spp. and *Mycobacterium* spp. The whole-genome sequence also served as the basis for carrying out microarray profiling to identify the differentially expressed genes in the bacterium. This led to the isolation of two promoters: *Pglx* and *PdsbA*, driving the expression of genes encoding glyoxalase and disulfide bond formation protein, respectively.

Microarray analysis also served as the basis for determining the expression pattern of 794 divergent gene pairs in *Gordonia* sp. IITR100. Several expression patterns were observed in these gene pairs and one promising gene pair controlled by divergent promoters was shortlisted for further experiments. Detailed molecular characterization of these two promising divergent promoters: *PmaiA* and *Phyd* (driving the expression of genes encoding maleate cis-trans isomerase and hydantoinase, respectively) was carried out. A dual-reporter promoter probe vector has been constructed in the study, which was utilized to demonstrate the co-expression ability of these divergent promoters. Key aspects of their regulation were also

studied, which revealed their biological significance. The evidence of the transcription of genes driven by the truncated derivatives of promoter fragments under different conditions serves as useful tools for fine-tuning their expression levels.

Based on the results of promoters obtained in the study, two strong promoters: *Phyd* and synthetic promoter were used to develop useful gene expression systems for therapeutic proteins in Actinobacteria. A shuttle expression vector was constructed to demonstrate the expression of single-chain analog of insulin (SCI-57). Further, a novel method for the expression of two subunits of Human Chorionic Gonadotropin (hCG) was developed using the divergent promoters working in opposite orientations, which pave the way for using Actinobacterial hosts for therapeutic protein production.

Ultimately, this study has led to the isolation of diverse promoters with different efficacy. The characterization of divergent promoters is noteworthy and provides several insights into their applicability. More studies on the identification of such systems will offer alternative approaches for co-expression of genes.

## सार

नवीन नियमन-योग्य प्रमोटर जीन अभिव्यक्ति प्रणालियों के निर्माण के लिए प्रभावी उपकरण के रूप में कार्य करते हैं। इस संदर्भ में विभिन्न प्रक्रिया-विशिष्ट अनुप्रयोगों में उपयोग के लिए विभिन्न शक्ति एवं नियामकता वाले प्रमोटरों की आवश्यकता होती है। प्रमोटर की कार्यक्षमता और विशेषताओं को समझना उनके उपयोग के लिए आवश्यक है। इस अध्ययन में गॉर्डोनिया प्रजाति ITR100 से पूर्व-पृथक्कृत स्थिर अवस्था प्रमोटर को एक मजबूत कृत्रिम प्रमोटर प्राप्त करने के लिए पुनरावृत्त यादृच्छिक उत्परिवर्तन द्वारा इंजीनियर किया गया जो ई. कोलाई में प्रमोटर के प्राकृतिक रूप की तुलना में स्थिर अवस्था प्रेरकता और उच्च शक्ति प्रदान करता है।

गॉर्डोनिया प्रजाति ITR100 से अतिरिक्त नवीन प्रमोटरों को अलग करने के लिए, संपूर्ण-जीनोम अनुक्रम निर्धारित किया गया, जिससे मुख्य प्रमोटर अनुक्रमों की पहचान की जा सकी। इसके आधार पर, मुख्य प्रमोटर अनुक्रमों का सर्वसम्मत अनुक्रम निकाला गया, जिससे संरक्षित विस्तारित -10 क्षेत्र की उपस्थिति का पता चला, जो एक्टिनोबैक्टीरिया के अन्य सदस्यों जैसे कि कोरिनेबैक्टीरियम प्रजातियों, रोडोकोकस प्रजातियों और माइक्रोबैक्टीरिया प्रजातियों में भी बताया गया है। माइक्रोएरे प्रोफाइलिंग द्वारा जीन की विभेदक अभिव्यक्ति प्रोफाइल का पता लगाया गया, जिसके कारण दो प्रमोटरों: P<sub>glx</sub> और P<sub>dsbA</sub>, की पहचान की गयी जो क्रमशः ग्लाइऑक्सालेज़ और डाइसल्फ़ाइड बंध निर्माता प्रोटीन को अभिव्यक्त करते हैं।

माइक्रोएरे प्रोफाइलिंग ने गॉर्डोनिया प्रजाति ITR100 में अपसरित जीन युग्म की अभिव्यक्ति प्रोफाइल को निर्धारित करने के आधार के रूप में भी कार्य किया। अभिव्यक्ति के कई प्रतिरूप देखे गए और आगे के अध्ययन के लिए एक जीन युग्म का चयन किया गया। दो आशाजनक अपसरित प्रमोटरों: P<sub>maiA</sub> और P<sub>hyd</sub>, जो क्रमशः मैलेट सिस-ट्रांस आइसोमेरेज़ और हाइडेंटोइनेज़ को अभिव्यक्त करने वाले जीन के अनुलेखन के लिए ज़िम्मेदार हैं, का विस्तृत निरूपण किया गया। अध्ययन में एक दोहरे

रिपोर्टर प्रमोटरधारी वाहक का निर्माण किया गया है, जिसका उपयोग अपसरित प्रमोटरों की सह-अभिव्यक्ति क्षमता को प्रदर्शित करने के लिए किया गया। उनके विनियमन के प्रमुख पहलुओं का भी अध्ययन किया गया, जिससे उनके जैविक महत्व का पता चला। विभिन्न परिस्थितियों में खण्डित किए गए प्रमोटर अंशों सहित अपसरित प्रमोटरों द्वारा संचालित जीन के अनुलेखन के प्रमाण उनके अभिव्यक्ति स्तरों को ठीक करने के लिए उपयोगी उपकरण के रूप में कार्य करते हैं।

अध्ययन में प्राप्त प्रमोटरों के परिणामों के आधार पर, दो मजबूत प्रमोटर: *Phyd* और कृत्रिम प्रमोटर का उपयोग एक्टिनोबैक्टीरिया में उपयोगी जीन अभिव्यक्ति प्रणाली विकसित करने के लिए किया गया। इन प्रमोटरों का उपयोग करते हुए एक अभिव्यक्ति वाहक का निर्माण इंसुलिन (SCI-57) के एकल-श्रृंखला अनुरूप की अभिव्यक्ति को प्रदर्शित करने के लिए किया गया था। विपरीत दिशाओं में काम कर रहे अपसरित प्रमोटरों का उपयोग कर मानव कोरियोनिक गोनाडोट्रोपिन (hCG) के दो उप-इकाइयों की अभिव्यक्ति के लिए एक नई विधि विकसित की गई, जो चिकित्सीय प्रोटीन उत्पादन के लिए एक्टिनोबैक्टीरियल मेजबानों का उपयोग करने का मार्ग प्रशस्त करती है।

अंततः, इस अध्ययन ने भिन्न-भिन्न प्रभावकारिता वाले नवीन प्रमोटरों को पृथक् किया है। अपसरित प्रमोटरों का अभिलक्षण उल्लेखनीय है और यह उनकी प्रयोज्यता में अंतर्दृष्टि प्रदान करता है। ऐसी प्रणालियों की पहचान पर आधारित अधिक अध्ययन जीन की सह-अभिव्यक्ति के लिए वैकल्पिक दृष्टिकोण प्रदान करेंगे।

# CONTENTS

Certificate	i
Acknowledgements	ii
Abstract	v
List of Contents	vii
List of Figures	x
List of Tables	xix
Abbreviations and Symbols	xx
<b>Chapter 1 Introduction and Objectives</b>	<b>1</b>
<b>Chapter 2 Literature Review</b>	
2.1. The promoter elements and transcription in bacteria	4
2.2. Structural attributes of promoters	7
2.3. Promoter types	8
2.4. Methods for isolation of promoters and their characterization	13
2.5. Reporter genes for characterization of promoters	17
2.6. Regulation of promoters	18
2.7. Stationary phase promoters in bacteria	21
2.8. Divergent promoters in bacteria	24
2.9. Mechanism of regulation of divergent promoters	27
2.10. Promoter engineering methods for improving strength to develop useful gene expression platforms in bacteria	29
2.11. Recombinant protein production in bacteria	35
2.12. The application potential of Actinobacteria	36
2.13. Expression systems for therapeutic proteins	37
2.14. <i>Gordonia</i> sp. IITR100	41

2.15. Research gaps	42
<b>Chapter 3 Materials and Methods</b>	
3.1. Bacterial strains, plasmids and primers used in the study	43
3.2. Media and growth conditions	53
3.3. Genomic DNA isolation	53
3.4. Random mutagenesis of the promoter fragment	54
3.5. Agarose Gel Electrophoresis	55
3.6. Polymerase Chain Reaction	56
3.7. DNA extraction from agarose gel	57
3.8. Plasmid isolation from transformed <i>E. coli</i> (manual method)	58
3.9. Plasmid isolation from transformed <i>E. coli</i> (kit method)	59
3.10. Cloning methodology	59
3.11. Preparation of <i>E. coli</i> chemical competent cells	67
3.12. Transformation protocol for <i>E. coli</i>	67
3.13. Preparation of electrocompetent cells of <i>Rhodococcus erythropolis</i> PR4 and electroporation	68
3.14. RNA isolation and cDNA synthesis	68
3.15. qRT-PCR	70
3.16. q-PCR	71
3.17. Promoter activity measurement	72
3.18. Whole genome sequencing	73
3.19. Microarray profiling	74

3.20. Resting cell assay	74
3.21. SDS-PAGE	74
3.22. Sample preparation and visualization of protein bands	75
3.23. Sample preparation and running on NuPAGE gels	76
3.24. Western Blotting	76
3.25. Microscopic techniques	77
3.26. Flow cytometry	78
3.27. Bioreactor cultivation	78
3.28. Bioinformatics analysis	78
<b>Chapter 4 Results and Discussion</b>	
4.1. Development of a strong stationary phase promoter-based gene expression system	80
4.2. Identification and isolation of promoters from <i>Gordonia</i> sp. IITR100	119
4.3. Molecular characterization of two divergent promoters <i>Phyd</i> and <i>PmaiA</i>	150
4.4. Development of gene expression system of insulin and hCG using the characterized promoters	196
<b>Chapter 5 Summary and Conclusions</b>	210
References	215
Appendix	242
Curriculum vitae	274

## LIST OF FIGURES

Figure No.	Title	Page No.
2.1	The transcription process in bacteria	5
2.2	Interaction between the promoter elements and different domains of RNA polymerase	7
2.3	Different classes of genes used for assessing promoter activity	18
2.4	The orientation of closely spaced promoters	25
2.5	Promoter engineering methods for various applications	32
4.1	Graph depicting the growth dependent $\beta$ -galactosidase activity of <i>E. coli</i> harboring plasmid pPOS6	81
4.2	Schematic diagram depicting the mutagenesis and cloning of promoter fragment	81
4.3	Graphs depicting the growth dependent $\beta$ -galactosidase activities of <i>E. coli</i> harboring plasmids p1.1-p1.4 obtained after first round of mutagenesis of stationary phase promoter	83
4.4	Alignment of promoter sequences of wild-type and P1.1	84
4.5	Graphs depicting the growth dependent $\beta$ -galactosidase activities of <i>E. coli</i> harboring plasmids p2.1-p2.7 obtained after second round of mutagenesis of P1.1	85
4.6	Alignment of promoter sequences of wild-type, P1.1 and P2.1	86
4.7	Graphs depicting the growth dependent $\beta$ -galactosidase activities of <i>E. coli</i> harboring plasmids p3.1-p3.3 obtained after third round of mutagenesis of stationary phase promoter	87
4.8	Alignment of promoter sequences of wild-type, P1.1 and P2.1	88
4.9	Secondary structures detected in the promoter regions	89
4.10	Bendability and curvature profiles of the promoters	90
4.11	A 1.5% agarose gel showing the RNA isolated from <i>E. coli</i> cells harboring pPOS21	92

4.12	Transcript levels of genes at the exponential and stationary phase of growth	93
4.13	A 10% SDS-PAGE showing expression of LacZ in whole cell lysate of <i>E. coli</i> cells containing pJJ1	94
4.14	SEM micrograph of <i>E. coli</i> harbouring pJJ1.	95
4.15	Bar graph depicting the OD <sub>600</sub> and $\beta$ - galactosidase activity of <i>E. coli</i> harboring pJJ1 at different temperatures	96
4.16	Bar graph showing the OD <sub>600</sub> and $\beta$ - galactosidase activity during prolonged growth-arrest conditions	97
4.17	Bar graphs showing $\beta$ -galactosidase activity of resting cells in the absence of nutrients	98
4.18	Strategy diagram for cloning <i>lacZ</i> under <i>P<sub>trc</sub></i> to construct plasmid pJJ3.	99
4.19	A 1% agarose gel showing clone confirmation of pJJ3.	100
4.20	Graph depicting the growth dependent $\beta$ -galactosidase activity of <i>E. coli</i> harboring plasmid pJJ3	101
4.21	Strategy diagram for cloning <i>lacZ</i> under T7 promoter to construct plasmid pJJ4	102
4.22	A 1% agarose gel showing clone confirmation of pJJ4	102
4.23	Graph depicting the growth dependent $\beta$ -galactosidase activity of <i>E. coli</i> harboring pJJ4	103
4.24	A 10% SDS-PAGE showing the expression of LacZ in whole cell lysates of <i>E. coli</i> harboring different plasmids	104
4.25	Bar graph depicting the activities of promoters obtained in the study with respect to <i>P<sub>trc</sub></i> and T7	105
4.26	Bar graph depicting the copy numbers of plasmids pJJ1, pJJ4 and pJJ3	106
4.27	Strategy diagram for cloning synthetic promoter in pEPR1 with GFP <sub>uv</sub> reporter gene to construct plasmid pJJ5	107
4.28	A 1% agarose gel showing construction of pJJ5	108
4.29	<i>E. coli</i> cells harboring pEPR1 (left) and pJJ5 (right) viewed under UV illumination	108

4.30	Graph depicting the growth profile and specific fluorescence intensity values of <i>E. coli</i> harboring pJJ5	109
4.31	Analysis of GFP <sub>uv</sub> expression in individual cells	110
4.32	Quantification of fluorescence intensity at different growth stages of <i>E. coli</i> harboring pJJ5	111
4.33	Strategy diagram for construction of an expression vector pJJ2 using the synthetic promoter	113
4.34	A 1% agarose gel showing clone confirmation of pJJ2	113
4.35	Strategy diagram for cloning <i>luxR</i> in pJJ2	114
4.36	A 1% agarose gel showing clone confirmation of pJJ6	115
4.37	Strategy diagram for cloning <i>dbrr</i> in pJJ2	115
4.38	A 1% agarose gel showing clone confirmation of pJJ7	116
4.39	A 12% SDS-PAGE showing the expression of LuxR and DBRR in whole cell lysates of <i>E. coli</i> harboring different plasmids	116
4.40	Expression of GFP <sub>uv</sub> in a 2 L bioreactor	117
4.41	A 0.8% agarose gel showing the genomic DNA isolated from <i>Gordonia</i> sp. IITR100	120
4.42	Gene ontology annotation of proteins showed their putative roles in biological processes, molecular functions and cellular components	121
4.43	Frequency of residues present at each position in the promoter region	122
4.44	The spacer length between the -35 and -10 region in promoters of <i>Gordonia</i> sp. IITR100	123
4.45	Growth curve of <i>Gordonia</i> sp. IITR100 with respect to time	124
4.46	A 1.5% agarose gel showing the RNA isolated from <i>Gordonia</i> sp. IITR100 at different time points	125
4.47	Bar graph depicting the genes upregulated or downregulated at OD <sub>600</sub> 1.5, 2.5 and 3.5 when expression at OD <sub>600</sub> 0.5 was used as control	126

4.48	Volcano plots for sorting of genes based on significant degree of fold change in expression and statistical significance	127
4.49	Heatmap of candidate genes showing the differential expression profile	128
4.50	Validation of microarray results by qRT-PCR	131
4.51	Heat map depicting the differential expression of 20 <i>glx</i> genes in <i>Gordonia</i> sp. IITR100	133
4.52	Putative promoter region of <i>Pglx5</i> showing the conserved nucleotides at -10 and -35 with the spacer region of 17 bp	134
4.53	A 1% agarose gel showing construction of pJJ14	135
4.54	<i>E. coli</i> cells harboring pEPR1 (left) and pJJ10 (right) viewed under UV illumination	135
4.55	Graph depicting the growth profile and specific fluorescence intensity values of <i>E. coli</i> harboring pJJ10	135
4.56	Strategy diagram for constructing shuttle vector pJJ11	137
4.57	A 1% agarose gel showing clone confirmation of pJJ11	138
4.58	Graph depicting the growth profile and specific fluorescence intensity values of <i>R. erythropolis</i> PR4 harboring pJJ11	139
4.59	Heat map depicting the differential expression of 3 <i>dsbA</i> genes in <i>Gordonia</i> sp. IITR100	140
4.60	Transcript levels of <i>dsbA1</i> in <i>Gordonia</i> sp. IITR100 determined by qRT-PCR	140
4.61	Putative promoter region of <i>PdsbA</i> showing the conserved nucleotides at -10 and -35 with the spacer region of 20 bp	141
4.62	A 1% agarose gel showing construction of pJJ12	142
4.63	<i>E. coli</i> cells harboring pEPR1 (left) and pJJ12 (right) viewed under UV illumination	142
4.64	Graph depicting the growth profile and specific fluorescence intensity values of <i>E. coli</i> harboring pJJ12	143
4.65	Strategy diagram for constructing shuttle vector pJJ13	144
4.66	A 1% agarose gel showing clone confirmation of pJJ13	144

4.67	Graph depicting the growth profile and specific fluorescence intensity values of <i>R. erythropolis</i> PR4 harboring pJJ13	145
4.68	Heat map of the differentially expressed 1588 divergent genes of <i>Gordonia</i> sp. IITR100 as revealed by microarray analysis	147
4.69	Graph showing the expression profile of the 794 divergent gene pairs of <i>Gordonia</i> sp. IITR100 at OD <sub>600</sub> values of 0.5, 1.5, 2.5 and 3.5)	148
4.70	Classification of monodirectional and divergent genes present in <i>Gordonia</i> sp. IITR100 and the categorization of divergent gene pairs based on microarray profiling	149
4.71	Bar graph depicting the fold change of expression levels of two divergent genes: <i>maiA</i> and <i>hyd</i> of <i>Gordonia</i> sp. IITR100	151
4.72	Gene coordinates of <i>hyd</i> and <i>maiA</i> in <i>Gordonia</i> sp. IITR100 and the function of hydantoinase and maleate cis-trans isomerase	152
4.73	Analysis of the conserved sequences in the 263 bp divergent promoter region between <i>hyd</i> and <i>maiA</i>	153
4.74	Strategy diagram for cloning of <i>PmaiA</i> and <i>Phyd</i> upstream to GFP <sub>uv</sub>	154
4.75	Expression of GFP <sub>uv</sub> by <i>PmaiA</i> and <i>Phyd</i> in <i>E. coli</i>	155
4.76	Strategy diagram for construction of shuttle vector of <i>PmaiA</i> -GFP <sub>uv</sub> (pJJ15)	156
4.77	A 1% agarose gel showing clone confirmation of pJJ15	156
4.78	Expression of GFP <sub>uv</sub> by <i>PmaiA</i> (left) and <i>Phyd</i> (right) in <i>R. erythropolis</i> PR4	157
4.79	Bar graph depicting the SFI of GFP <sub>uv</sub> driven by <i>PmaiA</i> and <i>Phyd</i> in (A) <i>E. coli</i> and (B) <i>R. erythropolis</i> PR4	158
4.80	Quantification of fluorescence intensity based on shift in peak of <i>E. coli</i> harboring pEPR1 (control), pJJ14 ( <i>PmaiA</i> ) and pBPM2 ( <i>Phyd</i> )	159
4.81	Strategy diagram depicting the construction of dual-reporter vector pJJ16	160

4.82	A 1% agarose gel showing clone confirmation of pJJ16	161
4.83	Strategy diagram depicting the cloning of divergent promoter region in dual-reporter vector pJJ16 to yield pJJ17	162
4.84	A 1% agarose gel showing clone confirmation of pJJ17	162
4.85	Co-expression of GFP <sub>uv</sub> and mCherry	163
4.86	Graph depicting the growth profile and specific fluorescence intensity values of <i>E. coli</i> harboring pJJ17 exhibiting co-expression of GFP <sub>uv</sub> and mCherry	164
4.87	Graph depicting the growth profile and specific fluorescence intensity values of <i>E. coli</i> harboring pJJ30 exhibiting co-expression of GFP <sub>uv</sub> and mCherry	165
4.88	Bar graph depicting the fold change in transcript of <i>gfpuv</i> and <i>mCherry</i>	166
4.89	Strategy diagram for construction of shuttle vector pJJ32	167
4.90	Graph depicting the growth profile and specific fluorescence intensity values of <i>R. erythropolis</i> PR4 harboring pJJ32 exhibiting co-expression of GFP <sub>uv</sub> and mCherry	168
4.91	Position of primers used for obtaining truncated derivatives of divergent promoters <i>Phyd</i> and <i>PmaiA</i> from both the orientation.	169
4.92	Graphs depicting the growth dependent expression profile of <i>E. coli</i> harboring plasmids pJJ17-25	170
4.93	Analysis of SFI of <i>E. coli</i> harboring plasmids pJJ21-29	171
4.94	Graphs depicting the SFI (A) and fold increase (B) in promoter activity of truncated promoter fragments as compared to the full-length promoter	172
4.95	Secondary structures in the 5'-UTR regions of (A) <i>hyd</i> and (B) <i>maiA</i>	173
4.96	Position of primers used for experimentally verifying the -10 and -35 region of <i>PmaiA</i> and <i>Phyd</i>	174
4.97	Bar graphs depicting the specific fluorescence intensity values of promoter fragments devoid of their respective -10	175

	and -35 regions (pJJ26-29) as compared to full-length promoter (pJJ17)	
4.98	Divergent promoter sequences with the central regulatory region containing binding sites of putative CRP and ArgR transcriptional regulators	176
4.99	Comparison of sequence of the CRP binding site with the <i>E. coli</i> consensus sequence	176
4.100	Multiple sequence alignment of CRP protein	177
4.101	Bar graph depicting the specific fluorescence intensity values of <i>E. coli</i> harboring pJJ17 when grown in minimal media containing either glucose or glycerol as carbon source	178
4.102	Bar graph depicting the specific fluorescence intensity values of <i>E. coli</i> harboring pJJ34 when grown in minimal media containing either glucose or glycerol as carbon source	179
4.103	Comparison of expression levels of GFP <sub>uv</sub> and mCherry in <i>E. coli</i> strains harboring pJJ17 grown in defined minimal media	181
4.104	Plasmids containing truncated divergent promoter fragments	182
4.105	Comparison of expression levels of GFP <sub>uv</sub> and mCherry in <i>E. coli</i> strain M182 harboring pJJ17-25 grown in minimal media containing (A) glucose and (B) glycerol	183
4.106	Comparison of expression levels of GFP <sub>uv</sub> and mCherry in <i>E. coli</i> strain M182 $\Delta$ CRP harboring pJJ17-25 grown in minimal media containing (A) glucose and (B) glycerol	184
4.107	Strategy diagram for constructing promoter fragment devoid of CRP binding site and construction of pJJ33	185
4.108	Comparison of expression levels of GFP <sub>uv</sub> and mCherry in <i>E. coli</i> strains harboring pJJ33 grown in defined minimal media	186
4.109	Comparison of expression levels of GFP <sub>uv</sub> and mCherry in <i>E. coli</i> strains harboring pJJ17 and pJJ33 in complex media	187

4.110	Comparison of sequence of the ArgR binding site with the <i>E. coli</i> consensus sequence	189
4.111	Bar graphs depicting the SFI of <i>E. coli</i> harboring plasmid pJJ17 grown in defined media with 0 or 0.1 mM arginine. (A) Glycerol as carbon source. (B) Glucose as carbon source	190
4.112	Strategy diagram for cloning Tetracycline resistance gene marker in pJJ17 to generate pJJ31	191
4.113	A 1% agarose gel showing clone confirmation of pJJ31	192
4.114	Comparison of promoter activities in <i>E. coli</i> wild-type (WT) cells (ArgR+) and delARG (ArgR-)	193
4.115	Mechanism of regulation of divergent promoters <i>PmaiA</i> and <i>Phyd</i>	195
4.116	Comparison of the activities of five promoters characterized in the study	197
4.117	Amino acid sequence of SCI-57	198
4.118	Strategy diagram for cloning synthetic promoter and <i>Phyd</i> upstream to <i>sci-57</i>	199
4.119	A 1% agarose gel showing clone confirmation of pJJ36	199
4.120	A 1% agarose gel showing clone confirmation of pJJ38	200
4.121	Expression of SCI-57 in <i>E. coli</i> Ori 2 (DE3) cells harboring plasmids pJJ35 (T7-INS); pJJ36 (SPP-INS); pJJ38 ( <i>Phyd</i> -INS) in sonicated extracts	201
4.122	Detection of insulin using Anti-insulin Mouse monoclonal primary antibody and Goat Anti-mouse IgG HRP conjugated secondary antibody	202
4.123	Strategy diagram depicting the construction of shuttle vector pJJ37	202
4.124	A 1% agarose gel showing clone confirmation of pJJ37	203
4.125	Strategy diagram depicting the construction of shuttle vector pJJ39	204
4.126	A 1% agarose gel showing clone confirmation of pJJ39	204

4.127	Detection of insulin using Anti-insulin Mouse monoclonal primary antibody and Goat Anti-mouse IgG HRP conjugated secondary antibody	205
4.128	Strategy diagram depicting the construction of pJJ42	206
4.129	A 1% agarose gel showing clone confirmation of pJJ42	207
4.130	Strategy diagram depicting the construction of pJJ43	207
4.131	A 1% agarose gel showing clone confirmation of pJJ43	208
4.132	Expression of pJJ40 (hCG- $\alpha$ ) and pJJ41 (hCG- $\beta$ ) in <i>E. coli</i> Ori 2 (DE3) cells harboring plasmids pJJ40 (hCG- $\alpha$ ), pJJ41 (hCG- $\beta$ ) and pJJ43 (hCG- $\alpha$ -divergent promoters-hCG- $\beta$ ) in sonicated extracts	209

## LIST OF TABLES

<b>Table No.</b>	<b>Title</b>	<b>Page No.</b>
2.1	Types of Sigma factors in different bacterial genomes	6
2.2	Constitutive promoters reported in bacteria	9
2.3	Inducible promoters reported in bacteria	11
2.4	Auto-inducible promoters reported from bacteria	13
2.5	Stationary phase promoters reported in bacteria	21
2.6	Divergent promoters reported in bacteria	25
2.7	Characteristics of promoters for various applications	29
2.8	Methods commonly employed for promoter engineering to develop gene expression system	33
2.9	Bacterial expression systems for insulin	39
2.10	Bacterial expression systems for hCG	41
3.1	List of bacterial strains used in the study	43
3.2	List of plasmids obtained commercially/gifted/used from previous studies	44
3.3	List of plasmids constructed in the present study	45
3.4	List of primers used in the study	49
3.5	Components of mutagenesis by Error-prone PCR	54
3.6	Reaction conditions for error-prone PCR	55
3.7	Components of a PCR reaction	56
3.8	PCR conditions	57
3.9	Ligation mixture components	60
3.10	Reaction components for cDNA synthesis	69
3.11	Reaction components for qRT-PCR	70
3.12	Reaction conditions for qRT-PCR	71
3.13	Components of SDS polyacrylamide gel	75

## ABBREVIATIONS AND SYMBOLS

$\Delta$	Delta/deletion
$\mu\text{g}$	Microgram
$\mu\text{l}$	Microlitre
Amp	Ampicillin
APS	Ammonium per sulphate
ArgR	Arginine Repressor
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool
bp	Base pair
$^{\circ}\text{C}$	degree Celsius
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary DNA
CRP	Catabolite repressor protein
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribose nucleotide triphosphate
EB	Elution buffer
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
Fig.	Figure
GFP <sub>uv</sub>	Green fluorescent protein
hCG	Human Chorionic Gonadotropin
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IPTG	Isopropyl $\beta$ -D thio galactopyranoside
Kan	Kanamycin
kDa	kilo Dalton
LA	Luria agar
LB	Luria Broth
MCC	Microbial culture collection
min	Minute
ml	Millilitre
mM	Milimolar
MTCC	Microbial type culture collection

NCBI	National Center for Biotechnology Information
OD	Optical density
ONPG	O-nitrophenyl $\beta$ -D galactopyranoside
Ori	Origami
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
<i>PdsbA</i>	Promoter transcribing gene encoding disulfide bond formation protein
<i>Pglx</i>	Promoter transcribing gene encoding glyoxalase
<i>Phyd</i>	Promoter transcribing gene encoding hydantoinase
<i>PmaiA</i>	Promoter transcribing gene encoding maleate cis-trans isomerase
PMSF	Phenylmethane sulfonyl fluoride
<i>Ptrc</i>	Trc promoter
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
qRT-PCR	Quantitative Reverse transcriptase PCR
RACE	Rapid amplification of c-DNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
SCI-57	Single-chain insulin analog-57
SDS	Sodium dodecyl sulphate
SFI	Specific fluorescence intensity
TAE	Tris-glacial acetic acid-EDTA
TBST	Tris buffered saline containing Tween-20
Tet	Tetracycline
Tris	Tris (hydroxymethyl) amino methane
UTR	Untranslated region
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
$\alpha$	Alpha
$\beta$	Beta