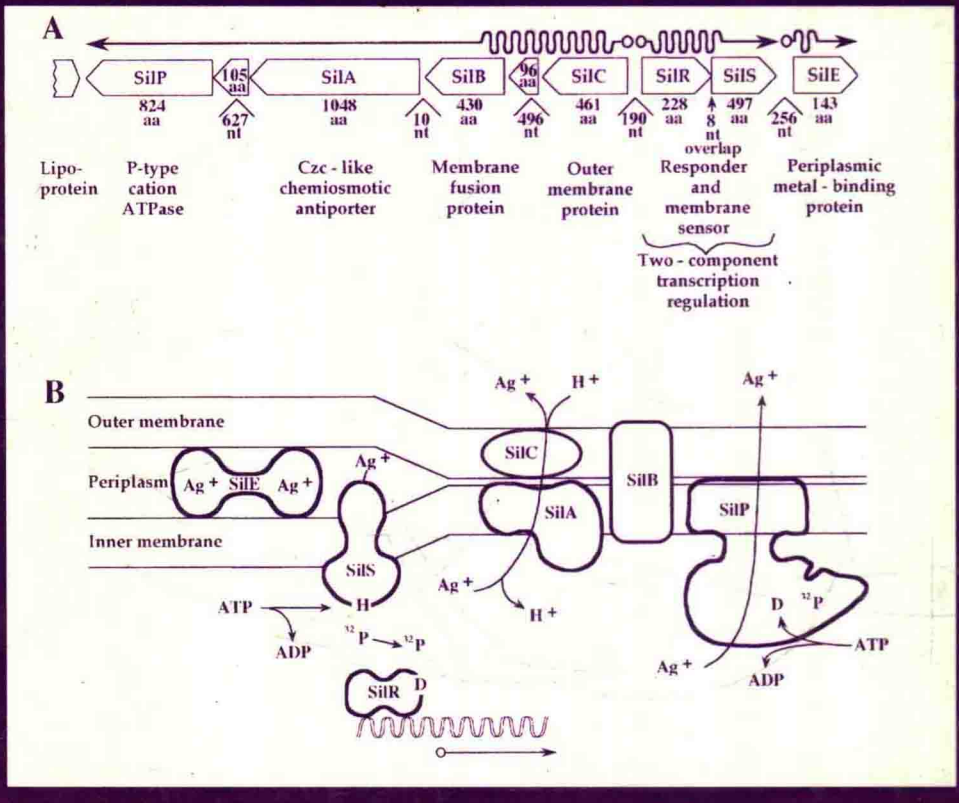
Industrial and Environmental Biotechnology

Edited by:
Nuzhat Ahmed, Fouad M. Qureshi, and Obaid Y. Khan



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Nuzhat Ahmed
Fouad M. Qureshi
Obaid Y. Khan

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Email: mail@horizonpress.com
Internet: www.horizonpress.com

Contributors

Ramla Abbas
Centre for Molecular Genetics
University of Karachi
Karachi-75270
Pakistan

M. Ahmad
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

M. N. Ahmed
National Institute for Biotechnology
and Genetics Engineering
P.O. Box 577
Jhang Road
Faisalabad
Pakistan

Nuzhat Ahmed
Centre for Molecular Genetics
University of Karachi
Karachi-75270
Pakistan
Email: a_nuzhat@yahoo.com

S. Ahmed
Department of Biological Sciences
Quaid-i-Azam University
Islamabad
Pakistan

Uzma Badar
Centre for Molecular Genetics
University of Karachi
Karachi-75270
Pakistan

Mussarat Bhatti
Agricultural Biotechnology Institute
NARC
Islamabad
Pakistan

T. A. M. Bridge
Div. Environ. and Applied Biology
Biological Sciences Institute
School of Life Sciences
University of Dundee
Dundee DD1 4HN
Scotland

N. C. Bruce
Institute of Biotechnology
University of Cambridge
Tennis Court Road
Cambridge CB2 1QT
U.K.
e-mail: n.bruce@biotech.cam.ac.uk

Ananda M. Chakrabarty
Dept. Microbiology and Immunology
University of Illinois
College of Medicine
835 South Wolcott Avenue
Chicago
Illinois 60612
USA
Email: Ananda.Chakrabarty@uic.edu

Sudha A. Chugani
Dept. Microbiology and Immunology
University of Illinois
College of Medicine
835 South Wolcott Avenue
Chicago
Illinois 60612
USA

N. A. Dahar
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

Geoffrey M. Gadd
Div. Environ. and Applied Biology
Biological Sciences Institute
School of Life Sciences
University of Dundee
Dundee DD1 4HN
Scotland UK
Email: g.m.gadd@dundee.ac.uk

M. M. Gharieb
Department of Botany
Faculty of Science
Menoufia University
Shebein El-Koom
Egypt

Michael A Gealt
Dean Eng. Mathematics and Science
Purdue University Calumet
2200 169th Street
Hammond
IN 46323
USA
Email: gealt@calumet.purdue.edu

Salma A. Ghori
School of Biomedical Sciences
Curtin University of Technology
GPO Box U1987
Perth
Western Australia 6845

Warren B Grubb
School of Biomedical Sciences
Curtin University of Technology
GPO Box U1987
Perth
Western Australia 6845
Email: W.Grubb@curtin.edu.au

A. Gupta
Dept. Microbiology & Immunology
University Of Illinois at Chicago
835 South Wolcott Avenue
Chicago
IL 60612-7344
USA
Email: agupta@UIC.EDU

Talat Haider
Department of Genetics
University of Karachi
Karachi-75270
Pakistan

A. Hameed
Department of Biological Sciences
Quaid-i-Azam University
Islamabad
Pakistan
E-mail: Hameed@microbe.sdnpk.undp.org

M. A. Javed
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

Umar Jawaid
Department of Genetics
University of Karachi
Karachi-75270
Pakistan

I. A. Khan
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

R. Khan
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

Obaid Y. Khan
Department of Genetics
University of Karachi
Karachi-75270
Pakistan

M. H. Khanzada
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

A. Khatri
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan.
Email: niatjam@hyd.paknet.com.pk

J.-F. Lo
Dept Microbiology & Immunology
University Of Illinois at Chicago
835 South Wolcott Avenue
Chicago
IL 60612-7344
USA

J. R. Lloyd
School of Biological Sciences
The University of Birmingham
Edgbaston
Birmingham B15 2TT
UK

L.E. Macaskie
School of Biological Sciences
The University of Birmingham
Edgbaston
Birmingham B15 2TT
UK
Email: L.E.Macaskie@bham.ac.uk

S. Parvez
National Institute for Biotechnology
and Genetics Engineering
P.O. Box 577
Jhang Road
Faisalabad
Pakistan
Email: shoukatparvez@yahoo.com

Le T. Phung
Dept. Microbiology & Immunology
University Of Illinois at Chicago
835 South Wolcott Avenue
Chicago
IL 60612-7344
USA

Azra Quraishi
Agricultural Biotechnology Institute
NARC Park Road
Islamabad
Pakistan
Email: abi-narc@yahoo.com

Fouad M. Qureshi
Centre for Molecular Genetics
University of Karachi
Karachi-75270
Pakistan

S. Raihan
Centre for Molecular Genetics
University of Karachi
Karachi-75270
Pakistan

M. I. Rajoka
National Institute for Biotechnology
and Genetics Engineering
P.O. Box 577
Jhang Road
Faisalabad
Pakistan

Deborah A. Rathbone
Institute of Biotechnology
University of Cambridge
Tennis Court Road
Cambridge CB2 1QT
U.K.

V. Saboo
Department of Bioscience and
Biotechnology
Engineering and Policy
Drexel University
Philadelphia
PA 1910
USA

J. A. Sayer
Div. Environ. and Applied Biology
Biological Sciences Institute
School of Life Sciences
University of Dundee
Dundee DD1 4HN
Scotland

R. Shahid
National Inst. Biotechnology and
Genetics Engineering
P.O. Box 577
Jhang Road
Faisalabad
Pakistan

S. H. Siddiqui
Plant Genetics Division
Nuclear Institute of Agriculture
Tandojam
Sindh
Pakistan

Simon Silver
Dept. Microbiology & Immunology
University of Illinois at Chicago
M/C 790 Room 703
835 South Wolcott Avenue
Chicago
IL 60612-7344
USA
Email: simon@UIC.EDU

V. Sobhon
Dept Bioscience and Biotechnology
Engineering and Policy
Drexel University
Philadelphia
PA 1910
USA

M. Sullivan
Dept. Bioscience and Biotechnology
Engineering and Policy
Drexel University
Philadelphia
PA 1910
USA

R. A. P. Thomas
School of Biological Sciences
The University of Birmingham
Edgbaston
Birmingham B15 2TT
UK

C. White
Div. Environ. and Applied Biology
Biological Sciences Institute
School of Life Sciences
University of Dundee
Dundee DD1 4HN
Scotland

Preface

Biotechnology is important in accessing environmental and industrial pollution problems, and also in providing potential "green" or environmentally friendly solutions to these problems. This is at least as true in developing countries such as Pakistan, as it is in more wealthy developed lands. History and recent experience tells us that countries such as Pakistan have major specific problems much in need of attention. There is also the major need for training young people at home so they will be able to apply the best of international standards to local needs.

The book chapters were originally based on presentations given at a key International Symposium in Biotechnology held in Karachi in 1998, however these have been extensively rvised and updated for publication in this book. This Symposium featured contributions from world-renown Asian and International scientists and it helped to make newer methods of genetic engineering and biotechnology available to Pakistani workers. Equally important, it made Pakistani needs more familiar to visitors, who then left with better understanding and a *readiness* to participate in future collaborative efforts.

There were a few continuing themes *in the Symposium that are reflected in this volume*. The environmental biotechnology section covers reports in two major categories of pollutants: organic compounds and metals. The organic compounds of industrial origin are frequently cyclic aromatic, and sometimes with nitrogenous or chloride substitutions. Dechlorination is the critical stage toward complete microbial biometabolism of xenobiotic compounds that never appeared in nature until synthesized and released by humankind. For this work, we have two reports by groups in North America and a report of efforts in Pakistan. It is clear that microbial bioremediation can work for many of these compounds. Sometimes it is less clear that engineers responsible for the decisions will be comfortable with microbes, enzyme and genes. One hopes that the Karachi meeting and this volume will bring together the people who developed *scientific* understanding with those *who* can apply and use it.

For genetic engineering, one needs to know the genes involved. A group from North America reported the current understanding of the genetic basis and molecular biology of microbial resistances to toxic metal ions. A few practical scale applications are coming into use for mercury pollution, both with microbial reduction removing Hg from wastewaters and microbial genes functioning in trees for "phytoremediation" of contaminated soils. Two groups from the United Kingdom describe their efforts in using microbial metabolism and microbial biomass to absorb toxic inorganic

ions and thus to remove the toxics from waste water solutions. Such methods are well developed and recent efforts include both laboratory scale studies and efforts at practical useful levels. Since the technologies can be of moderate cost and are widely accessible, these methods are particularly applicable in Pakistan *and many other countries*. A paper from our Pakistani colleagues concerns copper and chromate, both of which occur in effluents from leather-processing tanneries throughout the world, from Italy to Mexico to Pakistan. Bioaccumulation of copper and microbial reduction of chromate to insoluble $Cr(OH)_3$ are potentially practical methodologies. "Settling ponds" and use of endogenous resistant microbes (without need for specific genetic engineering) are attractive solutions that *are ready to* be applied at factory or village levels.

In addition to concerns with pollution and bio-solutions to clean-up problems, genetic engineering and biotechnology are important for production of useful materials, both in factories and in agriculture. A report from the United Kingdom on use of enzymes as biocatalysts to synthesize novel opiate derivatives of medical value introduced a large field for which we hope our Karachi colleagues will continue to contribute. The potentials seem almost endless. What is needed is to recognize a need and market, and then to adapt a biological (frequently microbiological) means to make the product.

Agricultural biotechnology and genetic engineering add still another dimension to the proceedings and to the potential for Pakistani solutions. We had three examples, firstly one of use of fungal microbes to convert abundant low-value molasses to higher value products. This is representative of a wide series of such uses of fungal cells in biotechnology, and how recombinant DNA/genetic engineering can advance the effort. The next two reports concerned Pakistani efforts to use tissue culture methods in the laboratory to improve sugar cane and potatoes crop *production* in the field.

Other reports at the Symposium that are not represented in this volume concerned other plants of economic importance. Basically for every plant grown by farmers, small as well as large scale, biotechnology and genetic engineering give promise for generating higher yields, disease resistance and other properties of value. There was little concern at the meeting with "GMO" foods, which seem to have caught attention in Europe in recent years. *The production of GMO crops has great potential to meet local needs, and scientific interaction such as occurred at this Symposium should help to avoid getting caught up in political disputes common in other countries.* Biotechnology and genetic engineering of crops can help Pakistani agriculture (*as well as agriculture everywhere*), but only with common sense and understanding.

The people who gathered in Karachi for the Symposium in 1998 shared one another's interests, enthusiasms and company for a week. We were like a family that wanted to share. With this volume as a record of what was started, we look forward to future collaborations, exchanges of people between laboratories and countries, and future gatherings in Karachi or elsewhere in Pakistan to continue what we have started. Insha'Allah!

Nuzhat Ahmed
Ananda Chakrabarty
Michael Gealt
Simon Silver

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1

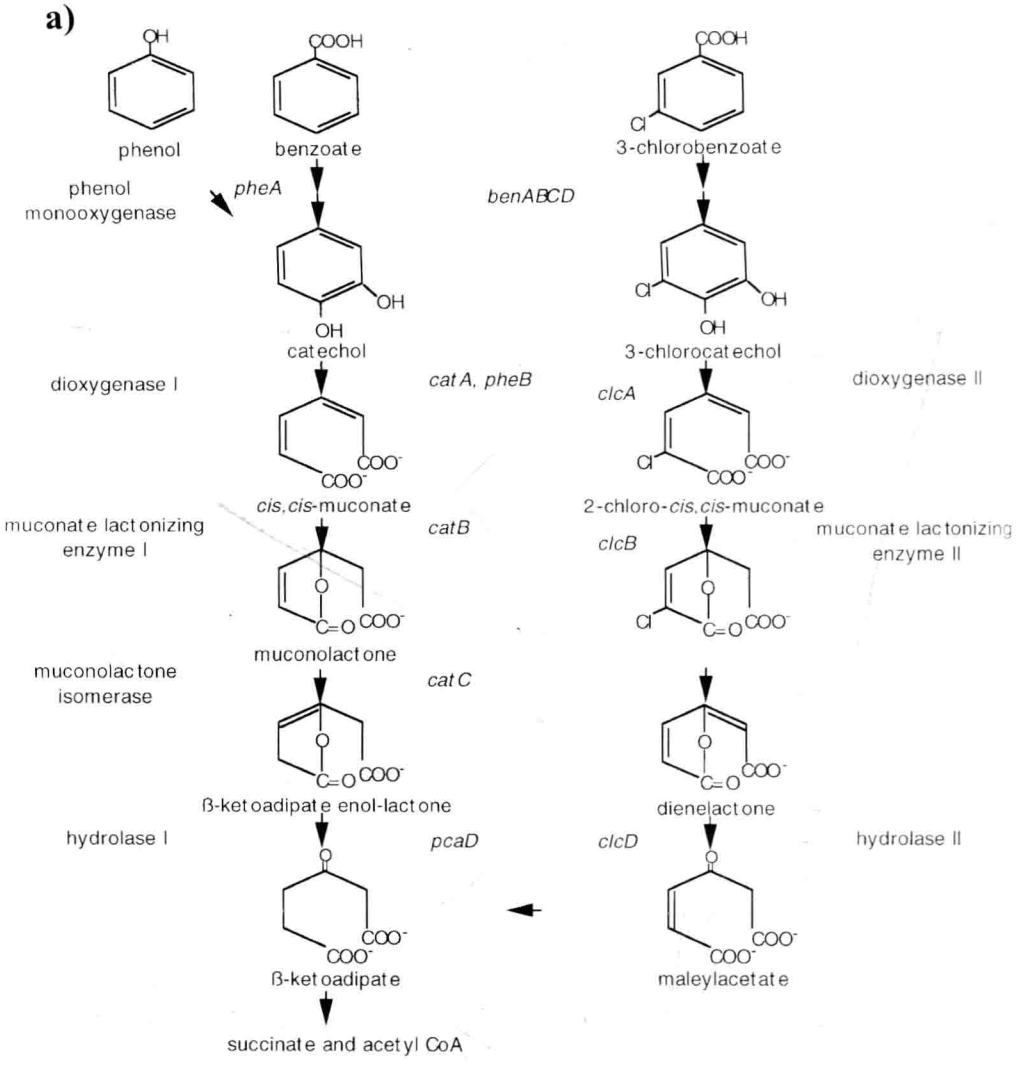
Molecular Mechanisms Regulating the Catechol and Chlorocatechol Biodegradative Operons

Sudha A. Chugani and Ananda M. Chakrabarty

ABSTRACT

The conversion of catechol and 3-chlorocatechol to tricarboxylic acid (TCA) cycle intermediates in *Pseudomonas putida* is mediated by the *catBCA* and *clcABD* operons respectively. The two operons are regulated by the LysR homologues CatR and ClcR with *cis,cis*-muconate and 2-chloromuconate as the respective inducers. CatR and ClcR have 32.5% sequence identity and 43% similarity at the amino acid level and a comparison of the *cat* and *clc* systems suggests that the two regulators utilize similar transcriptional activation mechanisms. *In vitro* transcription analyses showed that this mechanism involves interactions between the regulators and the α-carboxy terminal domain (α-CTD) of RNA polymerase. *In vitro* transcription studies done utilizing mutant RNA polymerases suggest that in case of CatR the α-CTD may also interact with the DNA at the promoter region. *In vitro* transcription studies using different *clcABD* promoter region fragments demonstrated that the α-CTD interacts with the ClcR dimer bound to the promoter proximal site.

Gel shift and footprinting analyses have shown that CatR has two binding sites in the *catBCA* promoter region. Another low affinity CatR binding site, the internal binding site (IBS) is located within the *catB* structural gene. Site directed mutagenesis studies demonstrated that this site repressed *catBCA* operon expression approximately 3-4 fold under inducing conditions *in vivo*. Footprinting and phasing experiments further indicated that the occupation of this site is mediated by cooperative interactions with CatR bound to the two upstream binding sites by looping of the intervening DNA.


a)
phenol
benzoate
3-chlorobenzoate
phenol monooxygenase
pheA
benABCD
catechol
3-chlorocatechol
dioxygenase I
catA, pheB
clcA
dioxygenase II
cis,cis-muconate
2-chloro-cis,cis-muconate
muconate lactonizing enzyme I
catB
clcB
muconate lactonizing enzyme II
muconolactone
muconolactone isomerase
catC
ß-ketoadipate enol-lactone
dienelactone
hydrolase I
pcaD
clcD
hydrolase II
ß-ketoadipate
maleylacetate
succinate and acetyl CoA
b)
catR
catB
catC
catA
ORF1
pheB
pheA
ORF2
clcR
clcA
clcB
(ORF3)
clcD

Growth of *P. putida* cells on succinate, citrate or fumarate results in repressed transcription from the *clcABD* promoter. It was further observed in *in vitro* transcription assays that fumarate, which is a TCA cycle intermediate, specifically abrogates *clcA* transcript formation. Both the catechol and chlorocatechol pathways feed into the TCA cycle; however, unlike the *clcABD* promoter the presence of succinate, citrate or fumarate did not affect expression from the *catBCA* promoter.

INTRODUCTION

Soil microorganisms, typically pseudomonads, are capable of utilizing a large number of natural and man-made compounds as carbon and energy sources (Harwood and Parales, 1996). The microbial dissimilation of synthetic compounds is becoming increasingly significant as man-made compounds are continually being released in the environment as herbicides/pesticides, solvents and various industrial byproducts. Many of these compounds are recalcitrant to microbial degradation through existing catabolic pathways and are harmful to the ecosystem. The breakdown of such compounds entails rapid adaptation of the resident microflora by either modification of the preexisting catabolic pathways or through gene recruitment. Artificially generating biodegradative pathways that allow the degradation of highly persistent compounds or expedite the dissimilation process may necessitate modeling new enzymes and regulators. It is therefore important to first study the existing biodegradative pathways, their organization, and their regulation. Along these lines, the study of the catechol-degradative *catBCA* operon, the chlorocatechol-degradative *clcABD* operon and the phenol-degradative *pheBA* operon of *Pseudomonas putida* has provided an insight into the molecular mechanisms of dissimilation of aromatic pollutants (Figure 1).

The *catBCA* operon is a representative of the central ortho/β-ketoadipate pathway mediating the catabolism of an aromatic compound. The *clcABD* operon is plasmid borne and presumably evolved from the chromosomal *catBCA* operon with modifications that allow the degradation of the chloroaromatic compound 3-chlorocatechol. The *pheBA* operon is flanked by DNA sequences similar to transposases of insertion elements and can be perceived as an excellent example of transposon-mediated rapid evolution wherein the evolution of only a couple of genes allows the degradation of the toxic chemical phenol. A comparison of the *cat*, *clc* and *phe* systems is interesting from the evolutionary viewpoint. It could be speculated that features conserved in the systems are important for efficient regulation and

Figure 1. a) Enzymes and intermediates of the benzoate, phenol, and 3-chlorocatechol biodegradative pathways. b) Organization of the genes of the *cat*BCA, *phe*BA and *clc*ABD operons.

functioning of the pathways and the differences between the pathways are important for conferring specificity.

A comparison of the DNA sequence and pathway enzymes suggests that the β-ketoadipate and modified ortho-cleavage pathways are closely related and that the catechol branch of the β-ketoadipate pathway is an evolutionary precursor of the modified ortho pathway for chlorocatechol degradation (Frantz and Chakrabarty, 1987; Ngai and Ornston, 1988). Regulation of the *catBCA* operon is mediated by CatR and the pathway intermediate *cis,cis*-muconate acts as an inducer (Rothmel *et al.*, 1990; Wheelis and Ornston, 1972). The *pheBA* operon allows the partial degradation of phenol in *P. putida* strain PaW85 (Kasak *et al.*, 1993). The monooxygenase encoded by *pheA* converts phenol to catechol, which is then funneled into the β-ketoadipate pathway (Figure 1a). In this case, the *pheBA* promoter is sufficiently similar to the *catBCA* promoter to allow regulation by the chromosomally encoded CatR, thus circumventing the need for the evolution of a separate regulator. The modified ortho pathway encoded by pAC27 for chlorocatechol degradation is controlled by ClcR, which presumably coevolved to ensure efficient transcriptional regulation (Coco *et al.*, 1993; Frantz and Chakrabarty, 1987). 2-chloromuconate, the intermediate of the 3-chlorocatechol pathway, is the inducer for the expression of the *clcABD* operon (McFall *et al.*, 1997c). Expression of the *catBCA* operon is induced ~100 fold when *P. putida* cells are grown in the presence of benzoate (Parsek *et al.*, 1994); expression of the *clcABD* operon is induced ~30 fold when *P. putida* cells are grown in the presence of 3-chlorobenzoate (McFall *et al.*, 1997c). CatR and ClcR are both LysR type transcriptional regulators (LTTRs) (Rothmel *et al.*, 1990; Schell, 1993).

CatR and ClcR, like all LTTRs, bind to their regulated promoters under both noninducing and inducing conditions. However, inducing conditions result in an altered footprinting pattern, changes in the DNA bending angle, and changes in the binding affinity. Two patterns have been described to explain the altered footprinting seen under inducing conditions. For CatR, presence of the inducer causes occupation of an additional adjacent binding site by increasing the number of bound monomers at both the *catBCA* and *pheBA* promoters. Under noninducing conditions, CatR occupies the Repression Binding Site (RBS), which is a 26 bp region extending from -79 to -54 relative to the *catB* transcriptional start site (Rothmel *et al.*, 1991). Upon inducer addition, the footprint extends to reflect the occupation of an additional adjacent 14 bp region (-47 to -34); this site is designated as the Activation Binding Site (ABS) and it partially overlaps the consensus -35 element (Chugani *et al.*, 1997; Parsek *et al.*, 1992). The decreased gel mobility of CatR-DNA complexes in the presence of the inducer can be attributed to the additional CatR protein that now occupies the ABS in addition to the RBS. Binding of CatR to the RBS elicits a bend in the DNA that is partially relaxed in the presence of *cis,cis*-muconate