1

**PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT**

PRECISIONSM RESOLUTION TARGETS

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**Plasmid pCU1 mediated killing of *Klebsiella oxytoca* -
molecular mechanism of KikA action.**

by

Martin Holčík, M.Sc.

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of**

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

1995

© copyright

1995, Martin Holčík

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

**THE AUTHOR HAS GRANTED AN
IRREVOCABLE NON-EXCLUSIVE
LICENCE ALLOWING THE NATIONAL
LIBRARY OF CANADA TO
REPRODUCE, LOAN, DISTRIBUTE OR
SELL COPIES OF HIS/HER THESIS BY
ANY MEANS AND IN ANY FORM OR
FORMAT, MAKING THIS THESIS
AVAILABLE TO INTERESTED
PERSONS.**

**L'AUTEUR A ACCORDE UNE LICENCE
IRREVOCABLE ET NON EXCLUSIVE
PERMETTANT A LA BIBLIOTHEQUE
NATIONALE DU CANADA DE
REPRODUIRE, PRETER, DISTRIBUER
OU VENDRE DES COPIES DE SA
THESE DE QUELQUE MANIERE ET
SOUS QUELQUE FORME QUE CE SOIT
POUR METTRE DES EXEMPLAIRES DE
CETTE THESE A LA DISPOSITION DES
PERSONNE INTERESSEES.**

**THE AUTHOR RETAINS OWNERSHIP
OF THE COPYRIGHT IN HIS/HER
THESIS. NEITHER THE THESIS NOR
SUBSTANTIAL EXTRACTS FROM IT
MAY BE PRINTED OR OTHERWISE
REPRODUCED WITHOUT HIS/HER
PERMISSION.**

**L'AUTEUR CONSERVE LA PROPRIETE
DU DROIT D'AUTEUR QUI PROTEGE
SA THESE. NI LA THESE NI DES
EXTRAITS SUBSTANTIELS DE CELLE-
CI NE DOIVENT ETRE IMPRIMES OU
AUTREMENT REPRODUITS SANS SON
AUTORISATION.**

ISBN 0-612-02952-2

Canada

Name Martin HOLCIK

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

MULTI-DISCIPLINARY

SUBJECT TERM

0307

SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523

Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language 0679
General 0289
Ancient 0290
Linguistics 0291
Modern
Literature 0401
General 0294
Classical 0295
Comparative 0297
Medieval 0298
Modern 0316
African 0591
American 0305
Asian 0352
Canadian (English) 0355
Canadian (French) 0593
English 0311
Germanic 0312
Latin American 0315
Middle Eastern 0313
Romance 0314
Slavic and East European

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion 0318
General 0321
Biblical Studies 0319
Clergy 0320
History of 0322
Philosophy of 0469
Theology

SOCIAL SCIENCES

American Studies 0323
Anthropology 0324
Archaeology 0326
Cultural 0327
Physical
Business Administration 0310
General 0272
Accounting 0770
Banking 0454
Management 0338
Marketing 0385
Canadian Studies
Economics 0501
General 0503
Agricultural 0505
Commerce Business 0508
Finance 0509
History 0510
Labor 0511
Theory 0358
Folklore 0366
Geography 0351
Gerontology
History 0578
General

Ancient 0579
Medieval 0581
Modern 0582
Black 0328
African 0331
Asia, Australia and Oceania 0332
Canadian 0334
European 0335
Latin American 0336
Middle Eastern 0333
United States 0337
History of Science 0585
Law 0398
Political Science 0615
General
International Law and Relations 0616
Public Administration 0617
Recreation 0814
Social Work 0452
Sociology 0626
General
Criminology and Penology 0627
Demography 0938
Ethnic and Racial Studies 0631
Individual and Family Studies 0628
Industrial and Labor Relations 0629
Public and Social Welfare 0630
Social Structure and Development 0700
Theory and Methods 0344
Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture 0473
General 0285
Agronomy
Animal Culture and Nutrition 0475
Animal Pathology 0476
Food Science and Technology 0359
Forestry and Wildlife 0478
Plant Culture 047
Plant Pathology 0480
Plant Physiology 0817
Range Management 0777
Wood Technology 0746
Biology 0306
General 0287
Anatomy 0308
Biostatistics 0309
Botany 0379
Cell 0329
Ecology 0353
Entomology 0369
Genetics 0793
Limnology 0410
Microbiology 0307
Molecular 0317
Neuroscience 0416
Oceanography 0433
Physiology 0821
Radiation 0778
Veterinary Science 0472
Zoology
Biophysics 0786
General 0760
Medical

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996

Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
General 0566
Audiology 0300
Chemotherapy 0992
Dentistry 0567
Education 0350
Hospital Management 0769
Human Development 0758
Immunology 0982
Medicine and Surgery 0564
Mental Health 0347
Nursing 0569
Nutrition 0570
Obstetrics and Gynecology 0380
Occupational Health and Therapy 0354
Ophthalmology 0381
Pathology 0571
Pharmacology 0419
Pharmacy 0572
Physical Therapy 0382
Public Health 0573
Radiology 0574
Recreation 0575

Speech Pathology 0460
Toxicology 0383
Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences
Chemistry 0485
General 0749
Agricultural 0486
Analytical 0487
Biochemistry 0488
Inorganic 0738
Nuclear 0490
Organic 0491
Pharmaceutical 0494
Physical 0495
Polymer 0754
Radiation 0405
Mathematics 0605
Physics 0986
General
Acoustics
Astronomy and Astrophysics 0606
Atmospheric Science 0608
Atomic 0748
Electronics and Electricity 0607
Elementary Particles and High Energy 0798
Fluid and Plasma 0759
Molecular 0609
Nuclear 0610
Optics 0752
Radiation 0756
Solid State 0611
Statistics 0463
Applied Sciences
Applied Mechanics 0346
Computer Science 0984

Engineering 0537
General
Aerospace 0538
Agricultural 0539
Automotive 0540
Biomedical 0541
Chemical 0542
Civil 0543
Electronics and Electrical 0544
Heat and Thermodynamics 0348
Hydraulic 0545
Industrial 0546
Marine 0547
Materials Science 0794
Mechanical 0548
Metallurgy 0743
Mining 0551
Nuclear 0552
Packaging 0549
Petroleum 0765
Sanitary and Municipal 0554
System Science 0790
Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

Carleton University
Ottawa, Canada K1S 5J7

30

Thesis contains black and white and/or coloured graphs/tables/photographs which when microfilmed may lose their significance. The hardcopy of the thesis is available upon request from Carleton University Library.

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis,

Plasmid pCU1 mediated killing of *Klebsiella oxytoca* -
molecular mechanism of KikA action.

submitted by
Martin Holčík, M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisor

External examiner

Carleton University

March 1995

Mojim rodičom

Abstract

When any self-transmissible conjugative plasmid of the group N is mated into *Klebsiella oxytoca*, but not *Escherichia coli*, the viability of the recipient is reduced 90-99%. This host specific phenotype is called Kik (killing of *Klebsiella*) and was studied using the plasmid pCU1. In the DNA of pCU1 there is a 500 bp region called *kikA* that has a role in determining this phenotype. Transposon insertions in *kikA* give rise to a Kik⁻ phenotype and to resistance to phage PRD1^R. The PRD1^R phenotype is observed in both *E. coli* and *Klebsiella*. The insertions in *kikA* do not affect the Tra⁺ phenotype in *E. coli*, however, the frequency of transfer in *Klebsiella* is reduced.

The Kik⁺ phenotype is not post-segregational; on the contrary, it is the continuous presence of pCU1 which is deleterious to the cells.

Two open reading frames of *kikA*, *orf104* and *orf70*, were separated by PCR and cloned into an expression vector. Expression of both *orfs* *in cis* or *in trans* was required for the Kik⁺ phenotype. However, expression of *orf104* alone was sufficient to restore the PRD1-sensitive phenotype and the Tra⁺ phenotype in *Klebsiella*. A polypeptide product of *orf104* has been identified. It is a soluble polypeptide which is processed and exported to the periplasm as a 8.9 kDa protein. Experiments with the radiolabelled phage PRD1 DNA suggest that this protein participates in the post-binding entry of the viral genome into the cytoplasm. The product of *orf70* is a 7.6 kDa protein found in the cytoplasm. The transcription of *kikA* in *Klebsiella* is positively controlled by an "augmenting" region whose precise location still needs to be determined.

The physiological mechanism of *kikA* action was explored by studying events

following *kikA* induction from an artificial promoter. Upon *kikA* induction the synthesis of host proteins, DNA and RNA was stopped. Transport of methionine, proline, and α -MG was reduced indicating that the overexpression of *kikA* in *Klebsiella* abolishes the generation of energy by dissipating membrane potential. This phenotype is similar to the mode of action of channel-forming bacteriocins. However, unlike these bacteriocins *kikA* must be expressed inside the host cell.

Acknowledgements

I would like to thank my supervisor Dr. V. N. Iyer for providing me with the opportunity to study at Carleton University in Canada. He gave me a once in a lifetime opportunity and I only hope that I fulfilled all his expectations. His continuous support, guidance and encouragement were very helpful and are greatly appreciated.

I want to extend my gratitude to Dr. Jo-Anne Dillon and Dr. George Carmody for being members of my advisory committee and for sharing their knowledge and experience with me.

I thank all my laboratory colleagues, past and present, whose support and constructive criticism helped in shaping this research and my own personality: Dr. Swapan Banerjee, Gaby Cherton, Clare Doxtater, Hong-Yeoul Kim, Paul Laroque, Suzanne Paterson, Margarita Rodríguez de González, and Beverly Wilkinson. My special gratitude goes to Dr. Peter Papp for teaching me how to think as a molecular biologist, for showing me many useful tricks with DNA and proteins and for always being patient with me. I thank Suzanne Paterson for synthesizing oligonucleotide primers.

I want to thank Dr. D. Sprott for introducing me to the secrets of membrane transport and membrane energy transduction, and to D. Watson for performing the protein microsequencing and mass spectrometer analysis.

While in Canada I met many people whose help was essential for this work. I want to express my deep gratitude to Mrs. Zoya Solod who took me, a total stranger, under her protective arms and created a home for me. My special thanks go to Katalin Bertenyi for being so supportive and understanding, and to her parents for making me feel welcome in their home.

Last but not least, I want to thank my parents and my brother for their continuous support despite the 6,000 km distance separating us.

My ability to study at Carleton University and to attend the Plasmid '94 conference was made possible by Dr. Iyer's research grant from the Medical Research Council of Canada.

Table of Contents

	Page
Title page	i
Acceptance Form	ii
Dedication	iii
Abstract	iv
Acknowledgements	vi
Table of Contents	vii
List of Abbreviations	ix
List of Figures	xv
List of Tables	xvii
Introduction	1
Literature Review	9
1. Killing from within the host cell	10
2. Killing from outside - colicins and microcins	27
Materials and Methods	33
Results and Discussion	67
Part 1. The killing of <i>Klebsiella</i> by plasmid pCU1 is not post-segregational	68
1.1 Construction of the pCU1 <i>lacZ</i> derivative	69
1.2 Bacterial matings with C600(pCU1 <i>lacZ</i>)	70
Part 2. Physiological consequences of the expression of <i>kikA</i>	76
2.1 Cloning of the <i>kikA</i> region under the control of the λ_{pr} promoter	77

2.2 Expression of <i>kikA</i> reversibly halts cellular growth	80
2.3 Effect of <i>kikA</i> expression on cellular metabolism	80
2.3.1 Effect of <i>kikA</i> expression on DNA, RNA and protein synthesis ..	80
2.3.2 Effect of <i>kikA</i> expression on the uptake of small molecules	83
Part 3. Genetic analysis of the <i>kikA</i> region	98
3.1 The role of the individual <i>orfs</i> of <i>kikA</i> in the KikA ⁺ phenotype:	
both <i>orfs</i> are needed for the KikA ⁺ phenotype	102
3.2 Isolation, partial purification and characterization of the Orf104 protein ..	108
3.3 Detection and characterization of the Orf70 protein	121
3.4 Expression of <i>kikA</i> - analysis of <i>kikA</i> putative promoter sequences	127
3.5 Location of the augmenting region	140
Part 4. Relationship between the different phenotypes conferred by the <i>kikA</i> region ..	147
References	167

List of Abbreviations

-	Absence of phenotype
A	Adenine
Ap	Ampicillin
ATP	Adenosine triphosphate
BSA	bovine serum albumine
bp	base pair(s)
C	Cytosine
°C	temperature in degrees Celsius
<i>caa</i>	gene encoding for the colicin A protein
<i>cai</i>	gene encoding for the colicin A immunity
<i>cal</i>	gene encoding for the colicin A lysis protein
<i>ccd</i>	coupled cell division
Ci	Curies(s)
cfu	colony forming unit(s)
Cm	Chloramphenicol
CsCl	Cesium chloride
Da	Dalton(s)
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DNA	deoxyribonucleic acid
<i>doc</i>	death on curing

DTT	dithiothreitol
ds	double stranded
EDTA	Ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
F'	F plasmid-bearing
<i>fbi</i>	fold-back inhibition
<i>flm</i>	F leading-region maintenance
G	Guanine
g	acceleration due to gravity
<i>gef</i>	gene expression fatal
gm	gram(s)
<i>gyr</i>	gene encoding for the DNA gyrase
hr	hour(s)
Hfr	High frequency of recombination
<i>hok</i>	host killing
Inc	incompatibility
IPTG	isopropyl- β -D-thiogalactopyranoside
kb	kilobase(s)
kDa	kilodalton
<i>kic</i>	killing of cell
<i>kid</i>	killing determinant
<i>kik</i>	gene(s) for killing of <i>Klebsiella oxytoca</i>

Kik	phenotype of <i>kik</i> genes
<i>kil</i>	gene(s) potentially lethal to <i>Escherichia coli</i>
<i>kis</i>	killing suppressor
Km	Kanamycin
<i>kor</i>	gene(s) required to override the effect of <i>kil</i> gene(s)
λ	bacteriophage Lambda
λP_R	bacteriophage Lambda right promoter
L	litre
<i>lacZ</i>	gene encoding the enzyme β -galactosidase
<i>lacI^r</i>	constitutively expressed lac repressor
LB	Luria broth
<i>let</i>	lethal mutation
M	molar (moles per litre)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mM	millimolar (10^{-3} moles per litre)
<i>mok</i>	mediation of killing
mRNA	messenger RNA
<i>mukB</i>	gene encoding 177 kDa protein involved in chromosome partitioning
MW	molecular weight
Nal	nalidixic acid

ng	nanogram(s)
nm	nanometre(s)
nt	not tested
O.D.	optical density
<i>orf</i>	open reading frame
<i>oriT</i>	origin of transfer replication
+	positive phenotype
+/-	presence or absence of phenotype
<i>par</i>	partitioning
PBS	phosphate-buffered saline
<i>pem</i>	plasmid emergency maintenance
PEG	polyethylene glycol
<i>phd</i>	prevent host death
pmol	picomole
<i>pnd</i>	promotion of nucleic acid degradation
<i>polA</i>	<i>E. coli</i> DNA polymerase I gene
<i>ppa</i>	gene encoding for pyrophosphatase in <i>E. coli</i>
P_{lac}	artificial hybrid promoter, IPTG inducible
R	resistance
R⁺	R plasmid-bearing
R⁻	devoid of R plasmid
<i>Rep</i>	replication region

Rif	rifampicin
RNA	ribonucleic acid
rpm	revolutions per minute
<i>rrnB</i>	transcriptional terminator (<i>E. coli</i> rRNA operon)
s	sensitive
ss	single stranded
SDS	sodium dodecyl sulphate
sec	second(s)
Sm	streptomycin
<i>sok</i>	suppressor of killing
Sp	spectinomycin
<i>srn</i>	stable RNA negative
T	Thymine
TAE	Tris-acetate, EDTA buffer
TBE	Tris-borate, EDTA buffer
Tc	tetracycline
TCA	trichloroacetic acid
TE	Tris-Cl, EDTA buffer
TES	Tris-Cl, EDTA, sodium chloride buffer
TIR	translation initiation region
Tn	transposon
<i>tra</i>	conjugative transfer gene(s)

Tra⁺	transfer proficient
Tra⁻	transfer deficient
U	unit(s)
μCi	microcurie(s)
μg	microgram(s)
μL	microlitre(s)
UV	ultraviolet
V	volt(s)
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
%	per cent
Δψ	electrochemical gradient (membrane potential)
<	less than
>	greater than

List of Figures

Figure	Description	Page
Figure 1.	Physical and genetic map of pCU1	6
Figure 2.	A schematic drawing of the PCR cloning	55
Figure 1.1.	Appearance of colonies of <i>K. oxytoca</i> with pCU1 <i>lacZ</i>	74
Figure 1.2.	Diagrammatic interpretation of the consequences of introducing pCU1 <i>lacZ</i> into <i>Klebsiella</i>	75
Figure 2.1.	Construction of the plasmid pMH5	79
Figure 2.2.	Growth of <i>K. oxytoca</i> carrying plasmid pMH5 after the temperature shift from 30°C to 42°C	81
Figure 2.3.	Growth of <i>K. oxytoca</i> carrying plasmid pMH5 after the temperature shift from 42°C to 30°C	82
Figure 2.4.	Effect of <i>kikA</i> expression on DNA, RNA and protein synthesis	84
Figure 2.5.	Effect of <i>kikA</i> expression on the uptake of L-methionine	85
Figure 2.6.	Schematic drawing of energy transduction across a membrane	88
Figure 2.7.	Effect of <i>kikA</i> expression on the uptake of α -MG	90
Figure 2.8.	Effect of <i>kikA</i> expression and KCN on the uptake of L-proline	91
Figure 2.9.	Effect of <i>kikA</i> expression on respiration	92
Figure 2.10.	Effect of <i>kikA</i> expression on the retention of L-proline	95
Figure 3.1.	Diagrammatic representation of the <i>kikA</i> region	100
Figure 3.2.	The nucleotide sequence of <i>kikA</i>	101
Figure 3.3.	Construction of the plasmid pMH104	103
Figure 3.4.	Construction of the plasmid pMH70	105

Figure 3.5.	Growth of <i>K. oxytoca</i> carrying various combinations of <i>kikA</i> orfs	107
Figure 3.6.	SDS-PAGE analysis of the Orf104 protein	109
Figure 3.7.	The predicted amino acid sequence of the Orf104 protein	110
Figure 3.8.	Comparison of the leader sequence of Orf104 with other proteins	113
Figure 3.9.	The hydrophobic profile of the Orf104 protein	114
Figure 3.10.	Mass spectrometer analysis of the Orf104 protein	117
Figure 3.11.	SDS-PAGE analysis of the Orf104 protein in the presence of mercaptoethanol	120
Figure 3.12.	The predicted amino acid sequence of the Orf70 protein	123
Figure 3.13.	SDS-PAGE analysis of the Orf70 protein	124
Figure 3.14.	Stability of the plasmids pPH4 and pPH0/1 in <i>K. oxytoca</i>	128
Figure 3.15.	Complementation of the Kik ⁺ phenotype	130
Figure 3.16.	Northern blot analysis of <i>kikA</i> RNA in <i>K. oxytoca</i>	132
Figure 3.17.	Construction of the plasmid pKK-P ₁	134
Figure 3.18.	Construction of the plasmid pKK-P ₂	135
Figure 3.19.	Construction of the plasmid pKK-P _{1,2}	136
Figure 3.20.	Analysis of the strength of the <i>kikA</i> promoters	138
Figure 3.21.	Identification of the augmenting region	145
Figure 3.22.	Construction of the plasmid pCU56Kan and pCU56Kan-G8	146
Figure 4.1.	Effect of <i>tonB</i> mutation on the infection of bacteriophage PRD1	157
Figure 4.2.	Bacteriophage PRD1 binding and DNA injection	159
Figure 4.3.	A role of Orf104 and Orf70 proteins in the Kik ⁺ and PRD1 ^s phenotypes: a model	163

List of Tables

Table	Description	Page
Table 1.	List of bacterial strains used	36
Table 2.	List of plasmids used	37
Table 3.	List of oligonucleotide primers used for PCR and/or sequencing	40
Table 1.1	Matings of C600(pCU1 <i>lacZ</i>) with <i>E. coli</i> and <i>K. oxytoca</i>	71
Table 3.1.	Biophysical properties of the Orf104 protein	111
Table 3.2.	The putative post-translational modifications of the Orf104 protein . . .	118
Table 3.3.	Biophysical properties of the Orf70 protein	126
Table 4.1.	Matings of C600(pCU1) and C600 (pCU403) in <i>E. coli</i>	149
Table 4.2.	Effect of <i>kikA</i> on conjugation in <i>Klebsiella</i>	150
Table 4.3.	The role of <i>kikA</i> in PRD1 ^s phenotype	153

Introduction

Bacterial plasmids are extrachromosomal elements capable of autonomous replication independent from the bacterial chromosome. Plasmid genes often encode optional characteristics which are selectively advantageous to bacteria in some environments but not others. The best known example of such genes is probably the antibiotic resistance genes carried on R (resistance) factors, also known as R plasmids. At the end of the 20th century, when almost all infectious diseases are treated with natural or synthetic antibiotics, the R plasmids still represent a serious threat due to the abilities of the plasmids to spread rapidly between different bacterial species, many of which are human or animal pathogens (Cohen, 1992).

Bacterial R plasmids are a very broad group of plasmids which have been studied extensively for several decades. According to their ability to occupy (coexist in) the same bacterial cell, plasmids are divided into several incompatibility (Inc) groups (Novick *et al.*, 1976). The plasmid determinants of compatibility are usually located close to that of plasmid replication, stable maintenance and copy number. All of these attributes are located in the plasmid replicon. Bacterial plasmids of the group N constitute a distinctive group of plasmids (Iyer, 1989). Plasmid pCU1 is a representative of the N incompatibility group, and its molecular biology has been in the past and continues to be the subject of detailed study at Carleton University.

Figure 1 shows the physical and genetic map of pCU1. It is a 39 kb plasmid carrying resistance genes for ampicillin (Ap^R), streptomycin (Sm^R) and spectinomycin (Sp^R). Plasmid pCU1 was originally isolated from the *Escherichia coli* K12 strain RM98' (Konarska-Kozłowska, and Iyer, 1981a). It was found that some RM98' strains carried a plasmid

aggregate (initially called RM98) which upon conjugation yielded two distinguishable plasmids. One, later called pCU1, exhibited all the criteria of an N group conjugative plasmid. The other, pCU2, has not been studied further. Plasmid pCU1 was later characterized (Konarska-Kozłowska and Iyer, 1981b) and was suggested as a reference IncN group plasmid. Its basic replicon (the smallest part of the plasmid able to replicate) is located within a 2 kb fragment and is composed of three clustered origins of replication (Kozłowski *et al.*, 1987; Krishnan and Iyer, 1990; Banerjee *et al.*, 1992; Kim *et al.*, 1994). The plasmid pCU1 is a broad-host-range plasmid since it can replicate and be maintained stably in a wide variety of Gram negative bacteria (*Escherichia coli*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Serratia*, *Klebsiella*) (Krishnan and Iyer, 1988; Kim, 1993). It has been suggested in the recent studies (Kim, 1993; Kim *et al.*, 1994; Papp and Iyer, 1995) that the broad-host-range of the plasmid replicon may be due to its rather complex structure. The three clustered origins of replication (*oriB*, *oriS* and *oriV*) have different requirements for their replication. Only the *oriB* and *oriS* can be utilized in the *polA* deletion mutant of *Escherichia coli* (Banerjee *et al.*, 1992). Furthermore, Krishnan *et al.* (1990) showed that Pol I-independence was correlated with the production of the 239 amino acid residue RepA protein encoded within the basic replicon. RepA binding sites were located within the functionally active *oriB* region which was able to replicate in a stable manner when RepA was supplied *in trans* (Papp and Iyer, 1995). In a separate study, Prince (1994) suggested that different origins of replication could be used in different hosts. It is plausible that functionally distinguishable origins in a small replicon may serve to confer broad host range. Although the host-range of the replicon is broad, that of the N *tra* system is even broader as shown by the

fact that linkage of the N *tra* system to the narrow-host-range replicon (p15) resulted in derivatives that entered but were suicidal in a much broader range of bacteria (Selvaraj and Iyer, 1984; Iyer, 1989). Plasmid genes required for conjugal transfer are located within a 15 kb *tra* region which is composed of eight complementation groups (A through H) (Thatte *et al.*, 1985b) and an origin of transfer, *oriT* (Paterson and Iyer, 1992). These genes are responsible for the synthesis and assembly of the conjugal pilus, DNA processing and transfer of DNA during bacterial mating. Also, bacterial hosts carrying pCU1 are sensitive to the filamentous bacteriophage IKe (Khatoon *et al.*, 1972) and the icosahedral phage PRD1 (Olsen *et al.*, 1974), both of which are pilus-specific bacteriophages.

Despite the fact that the plasmid pCU1 is a broad host range plasmid, it confers a rather peculiar phenotype called Kik⁺ (killing of *Klebsiella*). When *Klebsiella oxytoca* cells (previously *K. pneumoniae*, Yocum, 1989) were used as recipients in matings with *Escherichia coli* donors carrying pCU1, 90% to 99% of the recipients were killed (Rodríguez, 1981; Rodríguez and Iyer, 1981). Under similar conditions of mating, *E. coli* recipients did not display a reduction in viability, thus identifying the phenomenon as host-specific. Killing is not mediated by any substance which diffuses from *E. coli* to *Klebsiella* (Rodríguez and Iyer, 1981) and requires plasmid transfer by bacterial mating (Gill and Iyer, 1982; Thatte *et al.*, 1985a). Since killing was mediated by conjugation, the possibility that part of the *tra* region of pCU1 was involved in the killing mechanism was considered. By using Tn5 generated mutations within each of the transfer complementation groups, Rotheim *et al.* (1988) demonstrated that inactivation of each of these groups alone did not eliminate the Kik⁺ phenotype. In these studies, the plasmids with inactivated Tra functions were

transferred into *Klebsiella* with the help of a (Tra⁺) *oriT*⁻ derivative, pCU801. Genetic analysis of pCU1 using transposon and deletion mutagenesis provided evidence that there is one, and possibly two, separate regions of the plasmid required for the Kik⁺ phenotype. One region, known as *kikA*, is located next to the left end of *tra* on the pCU1 map (Figure 1). This region was identified by Kik⁻ Tra⁺ (transfer proficient) transposon Tn5 insertions (66, 167, 184)(Thatte *et al.*, 1985b), shown by nucleotide sequencing to be located at two sites (167=66)(Hengen, 1991). These Tn5 insertions were the only insertions which abolished the Kik⁺ phenotype without abolishing the Tra⁺ or IKe-sensitive phenotypes. Hengen (1991) analyzed the *kikA* region by molecular cloning, deletion analysis and nucleotide sequencing and localized the *kikA* region to a region of 500 base pairs. A fragment containing this region was placed under the transcriptional control of the inducible *tac* promoter. Upon induction with IPTG, killing of *Klebsiella oxytoca*, but not *E. coli*, was observed. This raised the possibility that in the natural plasmid pCU1, the *kikA* product(s) alone is sufficient for specifically killing *K. oxytoca* cells. Hengen's sequence of the *kikA* region implicated one or two open reading frames (*orf70* and *orf104*), and two putative promoters (P₁ and P₂) as being relevant to the phenotype. However, neither the function of these putative promoters nor the relevant polypeptides were identified.

Another aspect of the Kik⁺ phenotype is that all three Tn5 insertion mutants in *kikA* which were initially screened and found to be Kik⁻ were also found to be resistant to the phage PRD1, but sensitive to the phage IKe and to be Tra⁺ (Thatte *et al.*, 1985b). However, a mutant plasmid pCU21 (Rodríguez, 1981), obtained by chemical mutagenesis, was found to be Kik⁻ Tra⁺ but PRD1 sensitive. This indicates that the loci for the Kik⁺ and phage PRD1^s

Figure 1. Physical and genetic map of the plasmid pCU1 with emphasis on the regions relevant to this thesis. The functional complementation groups of the *tra* region are based on those of Thatte *et al.* (1985a,b). The *oriT* region (origin of transfer) is based on the sequence analysis of Paterson and Iyer (1992). The *kikA* region (enlarged below pCU1) has been shown (Thatte *et al.*, 1985a) and characterized (Hengen, 1991) previously. The coding sequences in the *kikA* locus (*orf104*, *orf70* and *orf80*) are indicated with arrows, the putative promoters (P₁ and P₂) are shown as boxes. Two black arrowheads (184, 66/167) indicate positions of Tn5 insertions leading to the Kik⁻ Tra⁺ PRD1^R phenotype. The *kikB* region (enlarged on the right) plays a role in the structural stability of pCU1 in the *Klebsiella* host (Rodriguez de González, 1994) and was defined by the different behaviours of the two pCU1 derivatives pCU109 and pCU101, clones in the vector pACYC184. Plasmid pCU403 (outermost broken line) is a *kikA*⁻ deletion derivative of pCU1 (Thatte *et al.*, 1985a). Rep is the replicon region and Ap, Sm, and Sp are regions specifying resistance to the respective antibiotics.

phenotypes are separable by mutation but that there is a functional relationship between them.

The presence of a second region, known as *kikB*, that augments the effect of *kikA* has been suspected because of differences in the efficiency or reproducibility of the Kik⁺ phenotype displayed by *E. coli* donors carrying the plasmid pCU101 (Kik^{+/+}) and pCU109 (Kik⁺) (Thatte, 1984). pCU101 has pCU1 coordinates 27.5 to 8.2 cloned into the *HindIII* site of the vector pACYC184 (Chang and Cohen, 1978) while pCU109 has coordinates 27.5 to 11.2 cloned into the same site, thus implicating coordinate 8.2 to 11.2 which was called the *kikB* region (see Figure 1). However, recent studies (Rodríguez de González, 1994) have suggested that the *kikB* region contributes to plasmid structural stability in *Klebsiella* and thus only indirectly to the Kik⁺ phenotype.

An unexpected and novel aspect of the Kik phenotype is that not only are the surviving *Klebsiella* carrying pCU1 immune to killing when used as recipients in a second round of mating but these derivatives are also immune when cured of pCU1. Gill (1985) and Rodríguez de González (1994) showed that such cured strains do not carry any detectable part of the plasmid and the latter also showed that the plasmid region needed for immunity to arise is separable from the *kikA* locus. This region and the mechanism by which immunity is established is under investigation.

The major objective of this study was to elucidate a mechanism of killing of *Klebsiella* at the molecular level. The previous observations of Rodríguez and Iyer (1981) have indicated that the Kik⁺ phenotype is not mediated by a substance that diffuses out from the plasmid-carrying donors and those of Hengen *et al.* (1992) showed that the killing of *Klebsiella* could be observed without conjugation and by events occurring from within *Klebsiella*. It will be

shown in the literature review that in *E. coli* there are two broad categories of killing from within cells, the first of which results in the killing of plasmid-free segregants in the population. A derivative of pCU1 was constructed and used to first discriminate between these two possibilities at the population level. The results are described and discussed in Part 1 of Results and Discussion. They do not support the possibility of post-segregational killing of plasmid-free cells in the population and indicate that the killing of *Klebsiella* requires the continued presence of the plasmid within the cells. Previous studies (Thatte *et al.*, 1985a; Hengen, 1991) have implicated the *kikA* region as a major component of the Kik phenotype. In the latter study it was shown that when the *kikA* region alone was cloned under the control of the *tac* promoter, the resulting derivative was stably maintained by *Klebsiella* but induction with IPTG resulted in loss of cell viability. This study therefore began with experiments that examined the physiological consequences of the overexpression of the *kikA* region in *Klebsiella*. These experiments are described and discussed in Part 2 of Results and Discussion. Since neither the *kikA* region under its natural promoter(s) nor the *kikA* region under the control of *tac* but without its induction appeared to result in a Kik⁺ phenotype, the study next focussed on a structural and functional analyses of the *kikA* region. The next objective was to address the questions of how the Kik⁺ phenotype and the bacteriophage PRD1-resistant phenotype are related and what the possible significance of the carriage of this region by the plasmid might be. The chapter that follows this introduction is a review of the literature on bacterial plasmids selected with the intention of providing information on other relevant examples of plasmid-determined killing of bacterial hosts and with the focus on the mechanisms of known killing systems rather than on their systematic description.

Literature Review

Bacterial cells commonly serve as hosts for plasmids and bacteriophages. Since both plasmids and bacteriophages are not capable of proliferation without the machinery of the bacterial host, their stable coexistence with the host would be expected to be symbiotic. However, this is not always the case. Bacteriophages are a good example of a parasitic behaviour, since often the killing and the lysis of their host is part of their life cycle. In contrast to bacteriophages, bacterial plasmids are generally thought of as stable extrachromosomal elements (Nordström and Austin, 1989). However, recent observations indicate that several bacterial plasmids carry genes capable of killing their host in a seemingly spiteful manner. The intention of this review is to give a comprehensive description of reasonably well-studied mechanisms employed by plasmid "killer genes" to destroy their target cell.

1. Killing from within the host cell.

Plasmids are normally inherited by both daughter cells when their host bacterium divides. If, however, the plasmid DNA did not replicate prior to cell division or the plasmid copies are physically joined to each other at the time of cell division, a plasmid-free daughter cell can be produced. To prevent this segregational loss, several independent mechanisms which ensure the stable maintenance of plasmids in the population have evolved. Some of these systems increase the likelihood of both daughter cells receiving a copy of the plasmid by selectively positioning plasmid DNA molecules and are often called "true" partitioning systems (Austin and Abeles, 1983; Ogura and Hiraga, 1983a; Davis et al., 1992; Hiraga, 1992; Williams and Thomas, 1992). Other stabilization systems are involved in the resolution

of the plasmid multimers into monomers, thus increasing the possibility of equal distribution of the plasmid molecules into the daughter cells (Austin *et al.*, 1981; Summers and Sherratt, 1984). Unlike these partitioning systems, the post-segregational killing systems found on some plasmids do not increase the likelihood of a daughter cell receiving the plasmid. On the contrary, plasmids carrying such genes are capable of selectively killing plasmid-free segregants after they are produced. This post-segregational killing was observed and described in the Inc FII plasmids R1 and F (Gerdes *et al.*, 1986a,b; Loh *et al.*, 1988) and there is also evidence that plasmids of the incompatibility groups P (RK2; Young *et al.*, 1985) and bacteriophage P1 (Lehnher *et al.*, 1993) encode gene products that may have an analogous role. Post-segregational killing systems always contain two components: a stable killer toxin and an unstable factor that prevents expression of the toxin or functions as an antidote to it. While the killer toxin is always a protein, the antitoxin is either antisense RNA (Gerdes *et al.*, 1986b; Loh *et al.*, 1988) or a protein (Jaffé *et al.*, 1985; Bravo *et al.*, 1988; Tsuchimoto *et al.*, 1988; Lehnher *et al.*, 1993).

The *ccd* locus (coupled cell division, Jaffé *et al.*, 1985; or control of cell death; Van Melderen *et al.*, 1994) of the F plasmid is probably the best characterized post-segregational killer system in which both components are proteins. The *ccd* region has two components: *ccdA*, (also called H or *letA*) which is a suppressor of inhibition, and *ccdB* (also called G, *letB* or *letD*) which codes for a lethal protein. Even though it was originally thought that the function of *ccd* is to couple cell division to the proliferation of the plasmid (Ogura and Hiraga, 1983b) it was shown later that the presumptive coupling is achieved by the killing of the plasmid-free segregants (Jaffé *et al.*, 1985). Jaffé *et al.* (1985) and later Hiraga *et al.* (1986)

showed that if the replication of the plasmid was prevented, the plasmid-free segregants became nonviable and formed long filaments. This phenomenon was explained by the nonviable segregant model (Jaffé *et al.*, 1985). When the replication of a thermosensitive-replication derivative of the plasmid was inhibited by a temperature shift, plasmid molecules were diluted by cell division and eventually a plasmid-free subpopulation of cells arose. These were, however, inhibited in their growth, formed filaments and became nonviable. It was speculated (Jaffé *et al.*, 1985) that this was due to the rapid decay of the CcdA protein which enabled the toxic *ccdB* gene product to kill the cells. Hiraga *et al.* (1986) showed in a separate experiment that this must indeed be the case, since the viability of the coexisting plasmid-free cells, which never carried the plasmid, was not affected. These experiments suggested that the killing action does not work by cell-to-cell contact and therefore the killing must be occurring from within.

Several studies have focused on the control and regulation of the *ccd* operon. It has been shown that expression of *ccdA* and *ccdB* is negatively controlled at the level of transcription (Tam and Kline, 1989a). The repression of the *ccd* operon was achieved only if both the CcdA and CcdB proteins were present. It was also shown (Tam and Kline, 1989b) that the CcdA and CcdB proteins form a complex even in the absence of the *ccd* operator. A recent study by Salmon *et al.* (1994) indicated that the domain of the CcdA protein responsible for autoregulation is different from the domain involved in the antidote effect. They constructed several missense CcdA polypeptides which lost their autoregulatory activities but retained the ability to antagonize the lethal activity of CcdB.

The CcdB protein acts by poisoning DNA-gyrase complexes (Bernard and Couturier,

1992; Maki *et al.*, 1992). The tetrameric A₂B₂ DNA gyrase is an essential cellular enzyme that catalyzes negative supercoiling of DNA (Gellert *et al.*, 1976). DNA gyrase introduces a transient double-strand nick in its substrate DNA and passes a double helix through the break in the direction which will decrease supercoiling of DNA. During the breaking and rejoining reaction, the 5' ends of the substrate DNA are covalently linked to the A subunit of gyrase (Bernard and Couturier, 1992). CcdB stabilizes the cleaved DNA-gyrase complex and does not allow resealing of the nicked DNA (Bernard *et al.*, 1993). These induced complexes then interfere with replication and transcription causing DNA lesions which in turn induce the SOS response and bacterial death (Salmon *et al.*, 1994). In this respect its action resembles that of the quinolone antibiotics and other antitumor drugs even though the CcdB protein is less efficient (Jaffé *et al.*, 1985). It was shown by *in vitro* and *in vivo* studies (Bernard and Couturier, 1992) that the CcdB protein is responsible for plasmid DNA linearization which suggests that it can promote gyrase-mediated double-stranded DNA breakage. As expected, an *Escherichia coli* strain resistant to CcdB (*ccdB*^r) was found to have a mutation in the A subunit of the DNA gyrase (Miki *et al.*, 1992). In this strain, the ability of CcdB to induce linearization of the plasmid DNA as well as SOS induction was suppressed. Interestingly, the ultraviolet light-triggered SOS induction was unaffected. Bernard and Couturier (1992) proposed a model in which the CcdB-promoted SOS induction is a consequence of DNA damage produced by a CcdB-gyrase-DNA complex rather than inhibition of gyrase activity. However, Maki *et al.* (1992) reported that an increased dosage of the wild type DNA gyrase overcame the growth inhibition of the CcdB product. This observation suggested that the target of CcdB protein is the DNA gyrase, particularly the A subunit. It was found that in the

CcdB-overproducing strain the DNA supercoiling activity was decreased to a nondetectable level (Maki *et al.*, 1992). This was due to the inactivation of DNA gyrase activity since the free DNA gyrase isolated from these strains was also completely inactivated. Addition of the CcdA protein to the extract of the CcdB-overproducing strain led to the full restoration of the gyrase activity (Maki *et al.*, 1992). These results indicated that the CcdA and the CcdB protein form an opposing pair in modulating the activity of the DNA gyrase. DNA gyrase plays a fundamental role in many cellular processes such as DNA replication, transcription, recombination and the resolution of daughter chromosomes (Miller *et al.*, 1990). An interesting possibility is that the CcdB protein also causes the inability of the bacterial chromosome to decatenate or to position the daughter chromosome which could lead to the observed filamentation and formation of anucleated cells (Miki *et al.*, 1992; Bernard and Couturier, 1992). Certain conditional mutations in the *gyrA* gene have essentially the same phenotype (Orr *et al.*, 1979; Hussain *et al.*, 1987a,b; Kato *et al.*, 1989). It is possible that the CcdB protein preferentially inhibits the decatenation activity of the DNA gyrase and the resulting higher topological order of the chromosomal DNA might interfere with proper chromosome segregation.

As mentioned above, the CcdB protein (11,700 Da) is necessary for filamentation and in the absence of the CcdA protein (8,700 Da) causes the death of the cell. CcdA and CcdB form a complex of about 69,000 Da *in vitro* (Tam and Kline, 1989b). Purified CcdA protein can protect wild type gyrase from CcdB poisoning (Maki *et al.*, 1992; Bernard *et al.*, 1993). It is believed that the CcdA protein interacts directly with the CcdB protein rather than with the DNA gyrase itself (Bernard *et al.*, 1993). Moreover, the CcdA protein was able to restore

the activity of the DNA gyrase isolated from the CcdB-overproducing strain (Maki *et al.*, 1992). In these experiments, the CcdB protein was found in the complex with dimers of DNA gyrase and the addition of stoichiometric amounts of the CcdA protein led to rejuvenation of the gyrase.

For proper functioning as a post-segregational killing system, the stabilities of the CcdA and CcdB proteins must be different. It was proposed by Jaffé *et al.* (1985) that in the plasmid-free cells, *de novo* synthesis of the Ccd proteins ceases and the CcdA protein is diluted enabling the CcdB protein to kill the cell. In recent work, Van Melderren *et al.* (1994) obtained evidence that the half-life of the CcdA protein is shorter than that of the CcdB protein. Using pulse-chase experiments, the half-life of the CcdA protein was estimated to be about 1 hour while the CcdB protein remained stable for over 2 hours. Interestingly, the stability of the CcdA protein improved in the Lon⁻ strain suggesting that Lon is the major protease responsible for the degradation of CcdA. It was also found that under conditions in which the CcdB protein is also produced, the stability of CcdA improved (Van Melderren *et al.*, 1994). This partial resistance to proteolysis was shown also for other unstable proteins degraded by the Lon protease (the Sula protein, Jones and McKnight, 1985; the RcsA protein, Stout *et al.*, 1991) and may be due to the fact that the motifs recognized by the protease are also involved in protein-protein interactions (Van Melderren *et al.*, 1994). An interesting observation is that the half-life of the CcdA protein is long when compared with other Lon targets such as the Sula, RcsA or λ N protein (1-2 minutes) (Van Melderren *et al.*, 1994). This, however, might be necessary considering the essential function of the antidote. Since Lon is a heat-shock protein, rapid changes in the surrounding environment could lead

to the degradation of the antidote even in the plasmid-harbouring cells and eventually to the killing of the cells. A degradation of the antidote that is too slow would, on the other hand, lead to the dilution of the toxin protein and thus the system would not be efficient (Van Melderren *et al.*, 1994).

The *ccd* operon of the plasmid F is not the only plasmid-borne post-segregational system with both the toxin and antidote appearing as proteins. The *parD* stability system of the plasmid R1 also codes for two genes: cytotoxin Kid (12 KDa) and the antidote protein Kis (9.3 KDa) (Bravo *et al.*, 1987; Ruiz-Echevarría *et al.*, 1991a). The ParD system is exactly conserved in the plasmid R100 where it has been described as the Pem system with the two genes *pemI* (identical to *kis*) and *pemK* (identical to *kid*) (Tsuchimoto *et al.*, 1988). It was shown in several studies that the mode of action of the ParD system is remarkably similar to that of the Ccd system. The *parD* operon is autoregulated at the level of transcription and both protein products are needed for this autoregulation in a manner similar to the autoregulation of the *ccd* operon of the plasmid F (Ruiz-Echevarría *et al.*, 1991b). Also, similar to *ccd*, the half-life of the toxic Kid (PemK) protein is longer than that of the antidote Kis (PemI) (Tsuchimoto *et al.*, 1992). The degradation of the PemI antidote was also shown to be exerted by the Lon protease (Tsuchimoto *et al.*, 1992). In the comparative study of *ccd* and *parD*, Ruiz-Echevarría *et al.* (1991b) showed that the ParD system of R1 and the Ccd system of F share not only functional but also sequence similarities. The CcdA and Kis proteins were found to be homologous at their N and C termini. However, despite these structural similarities, the CcdA and Kis proteins are not interchangeable. Also, the killer proteins of these two systems, the CcdB and Kid proteins, do not share any structural

similarities.

A functionally similar system was also found on the bacteriophage P1. As in the case of the *ccd* locus, there are two proteins involved in the post-segregational killing system of P1. The toxic protein Doc (126 amino acids) is prevented in its action in the cells which retained P1 by the antidote protein Phd (73 amino acids) (Lehnherr *et al.*, 1993). These two genes are organized in an operon (called addiction module) and the synthesis of Doc is translationally linked to that of Phd. Unlike killing by CcdB, expression of *doc* does not trigger SOS induction. Also, while killing by *ccd* or *pem* (*parD*) leads to filamentation, the cells that had lost P1 have a reduced size and a decreased capacity for SOS induction (Lehnherr *et al.*, 1993). There is no evidence to suggest that the DNA gyrase is the target of Doc protein; however, since the expression of *doc* does not trigger the SOS response it seems unlikely that the DNA gyrase is the target. As in the case of *ccd* and *pem* (*parD*), the shorter half-life of the antidote protein is due to its preferential degradation by protease. However, unlike the CcdA and PemI proteins, the Doc protein is degraded by the ATP-dependent serine protease ClpXp and not the Lon protease (Lehnherr and Yarmolinsky, 1994). Despite these differences the *doc/phd* addiction module shares the same organizational and structural parallels as the *ccd* or *pem* (*parD*) systems.

In other post-segregational killing systems the role of the antidote is played by antisense RNA. The best understood and characterized system of this type is the *parB* locus of the plasmid R1 (Gerdes, 1988). When the *parB* region was inserted into a plasmid with a temperature-sensitive replicon, the stabilization in the inheritance of such a construct at non-

permissive temperatures was observed (Gerdes *et al.*, 1985; Gerdes *et al.*, 1986a). Moreover, a large fraction of the population of plasmid-free cells was found to be non-viable. These non-viable cells, however, differed significantly from those observed with the *ccd* locus; the presence of *parB* did not induce filamentation, on the contrary, ghost cells were formed (Gerdes *et al.*, 1986a). The *parB* region was mapped to be within a 580 bp region and consists of two genes: *hok* (host killing) and *sok* (suppressor of killing) (Rasmussen *et al.*, 1987). The product of the *hok* gene is a small 52 amino acid hydrophobic polypeptide associated with the cellular membrane which, when overexpressed, causes cell death (Gerdes *et al.*, 1986b). It was observed that shortly after the synthesis of the Hok product, there is a rapid decrease in the rate of oxygen consumption and a decrease in the membrane electrochemical potential, $\Delta\psi$ (Gerdes *et al.*, 1986b). However, independent studies showed that the shortcircuiting of the membrane potential does not lead to inhibition of respiration and, *vice versa*, inhibition of respiration does not lead to the collapse of the membrane potential (von Meyenburg *et al.*, 1985; Gerdes *et al.*, 1986b). It was therefore proposed that the Hok polypeptide interacts with a component of the membrane, the inactivation of which will simultaneously lead to the observed changes in respiration and the membrane potential (Gerdes *et al.*, 1986b). The potential target for the Hok polypeptide was proposed to be some component of the respiratory chain since the inhibition of this function should lead to an inhibition of electron transport and proton pumping. The morphological changes associated with the expression of *hok* (ghost cell appearance) could be due to the permeabilization of the cell membrane (Gerdes *et al.*, 1986b). Due to the osmotic pressure and loosening of the peptidoglycan layer, water accumulates in the cell resulting in the expansion of the cell

volume and dilution of the intracellular material.

The expression of *hok* is regulated at the post-transcriptional level by the *sok*-encoded 67 nt antisense RNA which is transcribed in the opposite direction to *hok* (Gerdes *et al.*, 1990; Thisted *et al.*, 1994a; Thisted *et al.*, 1994b). It was shown that the target for the Sok-RNA is in the Hok mRNA leader region (SokT) (Thisted and Gerdes, 1992; Thisted *et al.*, 1994b). This region overlaps with the translation initiation region (TIR) of the third open reading frame *mok* (mediation of killing). It is likely that Sok-RNA inhibits *mok* translation by obstructing the *mok* TIR and preventing the entry of ribosomes to this region. The *mok* gene starts upstream of the *hok* gene and overlaps with *hok* out of frame. Translation of *mok* is very tightly regulated by Sok-RNA and the translation of *mok* is also a prerequisite for *hok* translation (Thisted and Gerdes, 1992). This genetical arrangement allows for the regulation of *hok* expression indirectly via *mok*.

Gerdes *et al.* (1990) found that the *hok* mRNA is very stable but the *sok* RNA decays rapidly. Also, a new truncated *hok* mRNA species was found and its formation was found to be negatively regulated by Sok-RNA. The truncated *hok* mRNA was found when the *sok* promoter was inactivated by site-specific mutagenesis or if rifampicin was added to cells containing the *hok/sok* locus. Addition of rifampicin also led to efficient expression of the Hok protein. On the other hand, addition of streptolydigin, which also led to the rapid decay of Sok-RNA, had no effect on the truncation of the *hok* mRNA or the synthesis of the Hok protein. It was therefore inferred that for the efficient translation of the *hok* mRNA, 3' -end processing is required (Gerdes *et al.*, 1990). In agreement with these findings, the truncated *hok* mRNA was found only in the plasmid-free cells (Gerdes *et al.*, 1990).

The regulation of *hok* mRNA translation by the antisense RNA seems very efficient but it poses one problem. In the plasmid-carrying cells, no *hok* translation is observed, implying very efficient inhibition by Sok-RNA, whereas in the plasmid-free cells the *hok* mRNA should be reactivated leading to the synthesis of the Hok protein. However, there has been no mechanism shown to operate in prokaryotes which can remove an antisense RNA from its target (Thisted *et al.*, 1994a). It has been shown recently that the *hok* mRNA is specifically cleaved at its 3' -end and that this processing leads to the translational activation of *hok* mRNA (Thisted *et al.*, 1994a, 1994b). Northern blot analysis of *in vivo* and *in vitro* produced Hok mRNA showed that there are two full-length mRNA species present: Hok mRNA-1 and Hok mRNA-2 (Thisted *et al.*, 1994a). While Hok mRNA-1 is very stable both *in vivo* and *in vitro*, Hok mRNA-2 decayed slowly. This decay was consistent with the appearance of the new, truncated Hok mRNA. A potential stem-loop structure was identified in the 3' -end of the full-length Hok mRNA-1 and was proposed to act as a barrier for 3' → 5' exonuclease degradation which could protect Hok mRNA-1 (Thisted *et al.*, 1994a). Using a cell-free system, Thisted *et al.* (1994a) showed that the full-length Hok mRNA-1 is translationally inactive whereas the truncated mRNA is translationally active and the translation inhibition motif, *fbi*, was mapped to the 3' -end region. Mapping of the full-length Hok mRNA indicated that the *fbi* region can interact directly with the *mok* TIR. Based on these results, Thisted *et al.* (1994a) proposed the following model for the regulation of post-segregational killing of plasmid-free cells. In a plasmid-carrying cell, the 3' -end *fbi* region binds to the 5' -end *mok* TIR region. This "closed" Hok mRNA is slowly processed from its 3' -end and the *fbi* region is removed. Removal of the *fbi* region will cause Hok mRNA to

open and to disclose the *mok* TIR region which is rapidly recognized and bound by the Sok-RNA. The binding of either *fbi* or Sok-RNA to the *mok* TIR region will prevent translation of the Hok protein. On the full-length closed Hok mRNA the binding of *fbi* to the *mok* TIR is slowly replaced by the binding of Sok-RNA (Thisted *et al.*, 1994b). This competitive binding of antisense RNA is significantly slower and ensures the presence of the pool of the full-length Hok mRNA in the cell. Once the Sok-Hok RNA complex is formed, it is recognized by RNase III and is rapidly degraded (Gerdes *et al.*, 1992). The binding of the *fbi* region to the *mok* TIR region is essential for the proper functioning of this system. Since the Sok-Hok RNA duplex is very rapidly degraded it would lead to a fast turnover of the *hok* mRNA in the cell. However, binding of *fbi* to *mok* TIR protects Hok mRNA from being degraded. In the plasmid-free cells, the pool of Sok-RNA decays rapidly since the Sok-RNA is highly unstable. The full-length Hok mRNA is slowly processed to its truncated active form. Since this truncated mRNA cannot be degraded in the absence of the Sok-RNA, it is efficiently translated. The synthesis of the Hok protein eventually leads to the killing of the cells. This system represents a unique "molecular memory" (Thisted *et al.*, 1994b).

Analogs of *hok/sok* were found also on other bacterial plasmids. Plasmid F carries the *smB* (Ohnishi, 1975) and *flm* (Loh *et al.*, 1988) loci, and the IncI α plasmid R483 and the IncB plasmid R16 each carry the *pnd* locus (Ohnishi and Akimoto, 1980; Akimoto and Ohnishi, 1982). These loci were originally identified due to their ability to induce membrane damage, RNase I influx, degradation of stable RNA and eventually cell killing upon addition of rifampicin. It was shown later by Nielsen *et al.* (1991) that these genes in fact mediate post-segregational killing of plasmid-free cells, are regulated by antisense RNAs, and that they

also share the same structural and functional organization as *hok/sok*. Thisted *et al.* (1994a) showed that the 3' -end processing of the killer mRNAs is essential for the activation of translation of these genes in a fashion similar to that of the Hok mRNA. It seems clear that these genes constitute a family of *hok*-homologous killer genes.

Surprisingly, homologs of the *hok* family killer genes were also found on the chromosome of *Escherichia coli*. The *relF* gene of the *relB* operon is related to the *hok* gene both structurally and functionally (Gerdes *et al.*, 1986). It encodes a 51 amino acid residue polypeptide which has 40% homology to the Hok protein. Induction of the *relF* gene leads to the same physiological response as the expression of the *hok* gene: cessation of respiration, dissipation of the membrane potential, appearance of ghost cells and cell death (Gerdes *et al.*, 1986). Another chromosomal gene, *gef*, was identified on the chromosome of *Escherichia coli* K12 (Poulsen *et al.*, 1989). The *gef* protein product was found to be structurally and functionally similar to the proteins encoded by *hok* and *relF*. Similarly to the Hok and RelF proteins, the Gef protein is a toxin protein of 50 amino acid residues and was found associated with the bacterial membrane (Poulsen *et al.*, 1991a). It was found that this protein forms dimers, however, the mutation which prevented the dimer formation had only a little effect on the toxicity of the Gef protein (Poulsen *et al.*, 1991a). It has been shown that even though the *gef* gene is transcribed constitutively, the Gef protein cannot be detected in the cells (Poulsen *et al.*, 1991b). The regulatory model for the control of the expression of *gef* was proposed to be similar to that of the regulation of the expression of *hok* (Poulsen *et al.*, 1991b). According to this model, translation of *gef* is coupled to the translation of the upstream open reading frame called *orf69* which is negatively regulated by the antisense RNA

Sof. This Sof-RNA can bind to the first three codons and the ribosome binding site of the *orf69* mRNA and thus prevents translation of *gef* (Poulsen *et al.*, 1991b). Two chromosomal mutations in *Escherichia coli* were identified which rendered cells resistant to the Gef protein (Poulsen *et al.*, 1992). The function of the proteins involved in Gef^R phenotype is unknown at present. Recently, a third operon of the *Escherichia coli* chromosome was identified to be homologous to the *hok* family. The functional and structural homolog of the *pem* locus was located at 60 minutes (*chpA*) and at 100 minutes (*chpB*) (Masuda *et al.*, 1993). *chpA* is located within the *relA* operon and *chpB* is located in close proximity to the *ppa* region. Both of these regions are believed to be involved in the stringent response suggesting a possible role of *chpA* and *chpB* in the stringent response (Masuda *et al.*, 1993).

Even though *relF*, *gef* and *chp* share structural and functional homology with the plasmid-borne killing systems of the *hok* family, the chromosomal killer genes, unlike their plasmid counterparts, were not shown to be able to stabilize plasmid inheritance (Poulsen *et al.*, 1989).

An entirely different killing system was also found to be associated with the chromosome of *Escherichia coli*. Two genes, *kicA* and *kicB* were identified upstream of *mukB*, a gene essential for chromosome partitioning (Feng *et al.*, 1994). KicA (antidote) and KicB (toxin) were found to be non-essential for the normal function of the cell. The *kicA* and *kicB* genes were cloned onto a cloning vector and their effect on the physiology of the host was investigated. Similarly to the *ccd* system of F, KicA can prevent killing by KicB presumably by protein-protein interaction with KicB. Preliminary experiments suggested that KicB interferes with protein synthesis resulting in the killing of the cell (Feng *et al.*, 1994).

Unlike other chromosome-borne killing systems, when *kicA-kicB* were transferred from the chromosome to a plasmid they were able to act in a "true" post-segregational killing manner by killing plasmid-free segregants (Feng *et al.*, 1994).

Although the presence of the killing genes on the plasmids can be explained in terms of increased fidelity of plasmid inheritance, their presence on the chromosome seems rather enigmatic. Interestingly enough, *gef* and *ref* analogues are conserved in several bacterial species including *E. coli* B and C, *Agrobacterium* and *Rhizobium*, suggesting that these genes play an important role in the physiology of the cell (Poulsen *et al.*, 1989).

Bacterial plasmids of the incompatibility groups P and N were found to carry an entirely different set of genes which are potentially lethal to their host. It was found that certain regions of the IncP α plasmid RK2 (indistinguishable from RP4) could not be cloned unless there were other regions of the same plasmid present *in cis* or *in trans* (Figurski *et al.*, 1982). These genes were designated *kil* and the genes which can prevent killing were called *kor* (killing override). Later experiments showed that the *kil/kor* genes constitute a network of coregulated genes which are also involved in the control of replication (Schreiner *et al.*, 1985; Jagura-Burdzy *et al.*, 1992; Motallebi-Veshareh *et al.*, 1992) and conjugation (Jagura-Burdzy *et al.*, 1992; Balzer *et al.*, 1992). There are four *kil* operons that have been identified: *kilA*, consisting of the genes *klaA*, *klaB*, and *klaC* (Goncharoff *et al.*, 1991; Saltman *et al.*, 1991), *kilB*, consisting of *trbB* and *trbC* (Thomson *et al.*, 1993; Larsen and Figurski, 1994), *kilC*, consisting of *klaA*, *klaB*, and *korB* (Larsen and Figurski, 1994), and *kilE*, consisting of *klaA-F* (Kornacki *et al.*, 1993). There are at least five *kor* genes (A, B, C, E, F) identified so

far (Larsen and Figurski, 1994). The *kil* and *kor* genes are organized into a *kil-kor* regulon which resides within a 26 kb region of RK2 (Larsen and Figurski, 1994). It is not known whether these genes are involved in the post-segregational killing of the plasmid-free cells or whether they contribute to the plasmid stability by some other mechanism. It was shown (Saltman *et al.*, 1991; Goncharoff *et al.*, 1991; Kornacki *et al.*, 1993; Thomson *et al.*, 1993; Larsen and Figurski, 1994) that unregulated expression of the *kil* genes leads to the killing of the host cells. The cells overexpressing the Kil proteins became elongated, had distorted outer membranes and their electron-dense materials were visible in the periplasmic spaces (Saltman *et al.*, 1991; Turner *et al.*, 1994). This cell-killing was always prevented if the corresponding *kor* region was present.

Incompatibility group N plasmids pKM101 and pCU1 were also shown to carry genes which are potentially lethal to their host. Even though there is no sequence homology to the *kil/kor* genes of RK2 and these genes cannot inhibit lethality of RK2 *kil* genes, Winans and Walker (1985) used the same terminology for pKM101 genes. Two *kil* genes (*kilA* and *kilB*) and two *kor* genes (*korA* and *korB*) were identified in the sequence of pKM101 and similar genes were identified in the plasmid pCU1 (Hengen, 1991; Rodríguez de González, 1994).

Several studies indicated that the function of the *kor* genes is to regulate expression of the *kil* genes on the level of transcription (Balzer *et al.*, 1992; Motallebi-Veshareh *et al.*, 1992; Jagura-Burdzy *et al.*, 1992; Kornacki *et al.*, 1993; Thomson *et al.*, 1993; Williams *et al.*, 1993; Moré and Winans, 1994). This type of regulation, however, is not expected for a system which would preferentially kill plasmid-free segregants since it cannot function as a "molecular memory" (Thisted *et al.*, 1994b). Interestingly, the *kilA* and *korA* sequences of

RK2 are part of the cryptic tellurite resistance transposon Tn521 (Grewal, 1990; Turner *et al.*, 1994) and the *korA* and *korB* sequences of RK2 are conserved throughout the incompatibility group P plasmids (Figurski *et al.*, 1982).

Similar potentially lethal genes were also described in plasmids associated with Gram-positive *Streptomyces* spp. Plasmid transfer in *Streptomyces* is associated with the formation of "pocks" corresponding to the areas of growth inhibition in the recipient strain (Bibb and Hopwood, 1981). Genetic studies with the plasmid pIJ101 identified four genes, *kilA*, *kilB*, *spdA*, and *spdB*, which are necessary for conjugal transfer and pock formation in *Streptomyces lividans* (Kendall and Cohen, 1987, 1988). Similarly to the *kil/kor* genes from RK2 or pKM101, *kil* genes of pIJ101 cannot be cloned unless their corresponding *kor* genes are present *in cis* or *in trans*. The *spdA* and *spdB* genes are responsible for the spreading of the plasmid in the mycelium after the transfer (Kendall and Cohen, 1988). The *kilA* (*tra*) gene is negatively regulated by the divergently transcribed *korA* gene, whose promoter overlaps the control region of *kilA* (Stein *et al.*, 1989). A second regulatory gene, *korB*, represses its own expression as well as the expression of *kilB* (Zaman *et al.*, 1992). Analogous *kil/kor* genes were also identified in the plasmid pSAM2 from *Streptomyces ambofaciens* and the multicopy plasmid pSN22 from *Streptomyces nigrifaciens* (Katoaka *et al.*, 1991). Conservation of these genes in *Streptomyces* plasmids and their involvement in conjugal transfer suggests that these genes might be involved in the regulation of the transfer processes.

Two more lethal phenomena are connected with conjugative plasmids. Lethal zygosis (Gross, 1963) and zygotic induction (Jacob and Wollman, 1961) are associated with plasmid transfer by conjugation. Lethal zygosis was observed as a reduced viability of the recipients in bacterial matings with *E. coli* Hfr donor cells. Recipients were inhibited in the synthesis of macromolecules, active transport across the membrane and the leakage of the cytoplasm was observed (Skurray and Reeves, 1973). Lethal zygosis is attributed to the fact that during prolonged mating, membrane rearrangement, which occurs in order to facilitate plasmid transfer can cause permeability of the recipient membrane followed by cell death (Skurray and Reeves, 1973; Ou, 1980). Zygotic induction is, on the other hand, caused by the transfer of a lysogenic bacteriophage inserted in the chromosome of the Hfr donor strain into a non-lysogenic recipient (Hayes, 1965). Since the recipient cells do not have the repressor protein which maintains the prophage in its lysogenic state, upon conjugal transfer phage genes are derepressed and the recipient cells are killed by the progeny phage.

2. Killing from outside - colicins and microcins.

All the plasmid-encoded toxic genes which were discussed in the previous chapter had one common feature: they all killed from within the host cell. Bacterial colicins and microcins represent a large group of plasmid-encoded toxic proteins and polypeptides which, however, do not have a deleterious effect on the host bacterium. They are produced by bacterial strains carrying specific plasmids called colicinogenic factors (Morlon *et al.*, 1983). Synthesis of colicins may be induced by mutagenic agents as well as by thymine starvation. More than 20 colicins and several microcins have been described in *Escherichia coli* and other non-

sporulating bacteria (Konisky, 1982; Baquero and Moreno, 1984; Riley, 1993). Bacteriocins (colicins and microcins) are abundant in natural populations of *Escherichia coli*, since 30% of the *E. coli* isolates from human and domestic animals possess one or more bacteriocins (Riley and Gordon, 1992). Although the ecological role of colicins is unclear, several studies suggest that they play a role in bacterial competition and invasion (Smith, 1974; Chao and Levin, 1981).

Bacteriocins are usually grouped according to their molecular weight: microcins (MW < 5000 Da) and colicins (MW > 5000 Da) (Baquero and Moreno, 1984). For the purpose of this review, however, bacteriocins will be described according to their mode of action.

The target for many bacteriocins is a component of the protein-synthetic machinery of the cell. Colicin E3 and cloacin DF13 are probably the most thoroughly understood bacteriocins with this mode of action (Konisky, 1982). It has been shown for *Escherichia coli* cells treated with colicin E3 that isolated 70S ribosomes were defective in their ability to support *in vitro* protein synthesis (Nomura, 1963). The damage was further localized to the 16S RNA of the 30S ribosomal subunit. It was shown both *in vivo* (Bowman *et al.*, 1971) and *in vitro* (deGraaf *et al.*, 1973) that this was due to the cleavage of 49 nucleotides at the 3'-end of the 16S RNA. Similar observations were recorded with cloacin DF13-treated *Enterobacter cloacae* cells (deGraaf *et al.*, 1971, 1973). These results indicated that colicin E3 may act as a ribonuclease or that it may induce the latent ribonuclease activity of some ribosomal protein(s). It was shown later (Ohno and Imahori, 1978) that the degradation of rRNA is due to colicin E3 itself and that colicin E3, and by comparison also other related colicins, have endogenous RNase activity. Other bacteriocins such as microcin A15 and C7

also seem to inhibit synthesis of proteins, while the target of colicin E2 and microcin B17 is DNA replication (Konisky, 1982; Baquero and Moreno, 1984).

It will become apparent in the Results and Discussion section of this thesis that there is a certain functional similarity between the *kikA* mode of action and the action of channel-forming colicins. Therefore the mode of action of channel-forming colicins is described in greater detail.

There are several colicins and microcins such as colicin A (Lazdunski *et al.*, 1988), colicin E (Fields and Luria, 1969a,b), colicin Ia (Gilchrist and Konisky, 1975), colicin Ib (Weaver *et al.*, 1981), colicin K (Schein *et al.*, 1978), colicin V (Yang and Konisky, 1984), lactacin F (Abee *et al.*, 1994), microcin E492 (Lagos *et al.*, 1993), nisin A (Kordel and Sahl, 1986) and pediocin PA-1 (Chikindas *et al.*, 1993) which form ion-permeable channels in bacterial cytoplasmic membranes. Even though these bacteriocins do not share any homology with each other and they originate from a wide variety of bacteria, their mode of action is very similar and can be described using the example of colicin A.

Colicin A is a large bacteriocin with a molecular weight of 62,989 Da (Morlon *et al.*, 1983). It is a plasmid-encoded protein encoded by the multicopy plasmid pColA. There are three plasmid genes required for the proper functioning of colicin A: the gene for colicin A, *caa*, the gene for colicin A immunity, *cai*; and the gene product required for colicin release, *cal* (Lazdunski *et al.*, 1988). Nucleotide sequencing analysis revealed that *caa* and *cal* form an operon, and that *cai* is located between these two genes and is transcribed in the opposite direction from its own promoter (Lazdunski *et al.*, 1988). Colicin A is produced in very large amounts and can account for more than 50% of the total cellular protein (Morlon *et al.*,

1988). Even though colicin A is a secreted protein, it is not synthesized as a precursor (Cavard *et al.*, 1981). The product of the *cal* gene (colicin A lysis) is necessary for the release of colicin A. The structures of the known Col plasmid release proteins were compared and they shared significant homology (Lazdunski *et al.*, 1988). This homology is conserved in the N-terminal signal sequence and the cleavage site which resembles the consensus lipoprotein modification sequence. Comparison with ColE2, ColE3 and ColN lysis proteins (Cal) suggests that the C-terminal parts of the lysis proteins are not important for their function (Lazdunski *et al.*, 1988). The detailed mechanism of colicin release is not known. However, it is believed that the Cal protein must be modified by a lipid (Cavard *et al.*, 1987) and the process of colicin A release is non-specific with respect to colicin itself and depends solely on the expression of the colicin A lysis protein Cal (Lazdunski *et al.*, 1988). Immunity to colicin A is provided by the Cai protein. It is a membrane spanning protein which specifically interacts with the C-terminal domain of colicin A, thus protecting the cell against the effect of the external colicin A (Lazdunski *et al.*, 1988). The Cai protein, however, cannot protect the cell against heterologous colicins with identical modes of action (Bishop *et al.*, 1985).

Once colicin A is released from the producing cell it can bind to the specific receptors of the susceptible cells (Konisky, 1982). Colicin A binds to the outer membrane proteins BtuB and OmpF (Letellier, 1992). Upon binding to the receptor, colicin A is translocated through the envelope using the transport machinery of the host cell encoded by the *tol* Q, R, A, B gene cluster (Cardelli and Konisky, 1974; Sun and Webster, 1986, 1987; Traub and Braun, 1994). The colicin-receptor interaction is energy-independent, and even the translocation of the colicin can be achieved in de-energized cells indicating that the functional membrane

potential is not required (Bourdineaud *et al.*, 1990).

Kinetic analysis of the efflux of cytoplasmic K⁺ induced by colicin A indicates that colicin A forms a channel in the bacterial membrane (Bourdineaud *et al.*, 1990). It has been shown that addition of one colicin A molecule to one bacterium induced a rapid efflux of internal K⁺. This efflux occurs independently of the K⁺ transport system and seems to be channel-mediated (Letellier, 1992). These experiments also suggested that there is no cooperativity between colicin A molecules and presumably each molecule forms a single channel. It has been shown (Martinez *et al.*, 1983 in Lazdunski *et al.*, 1988) that it is the 48 amino acid C-terminus which is involved in pore formation. The single-channel conductance of the colicin A pore was shown to be almost identical to that of colicin E1 (Lazdunski *et al.*, 1988). Several models were proposed for the structure of the colicin A channel (Patils *et al.*, 1985; Lazdunski *et al.*, 1988). Based on conductance measurements and site-directed mutagenesis of colicin A, it seems that there are three helices which span the membrane bilayer and two more helices which form the mouth of the pore (Lazdunski *et al.*, 1988).

Addition of colicin A to the cell suspension results in the partial depolarization of the inner membrane. It was observed, however, that the steady state of $\Delta\psi$ (membrane potential) (about 70 mV, negative inside) was reached suggesting that the channel closes when the threshold is attained (Letellier, 1992). This poses a question of how colicin A kills bacteria since it was shown in independent experiments (Kinoshita *et al.*, 1984) that reduced membrane potential does not cause cell death. It has been shown for other channel-forming colicins (Fields and Luria, 1969b) that upon the addition of the colicin to the cell suspension, up to 80% of the internal ATP was depleted in less than 2 minutes. This observed ATP

depletion, however, was not due to the leakage of internal ATP. It was suggested by Letellier (1992) that the decrease in ATP is the consequence of the efflux of inorganic phosphate through the colicin channel and that this massive drop in the internal ATP concentration is causing cell death. In the case of channel-forming microcin D140, D15 and E492 (Baquero and Moreno, 1984; Lagos *et al.*, 1993) the depletion of ATP is also accompanied by the increased activity of bacterial F_0F_1 ATPase. Since this effect was not observed in the ATPase null mutants it was suggested (Duro *et al.*, 1979) that this increased activity of the bacterial ATPase is responsible for the depletion of internal ATP.

Materials and Methods.

Bacterial strains, plasmids and bacteriophages.

The bacterial strains used in this study are listed in Table 1. The plasmids used and recombinant plasmids constructed in this study are listed in Table 2. The bacteriophage PRD1 was described (Olsen *et al.*, 1974) and was obtained from R.H. Olsen.

Media, chemicals and enzymes.

Luria-Bertani (LB) broth (1% w/v tryptone (Difco), 1% w/v NaCl and 0.5% w/v yeast extract (Difco) with or without 1.5% agar (w/v, Difco) was used as a standard organic medium. The minimal media used was that of Davis and Mingioli (1950) and was prepared by mixing sterilized solutions of organic salts (10.5 g/L K_2HPO_4 , 4.5 g/L KH_2PO_4 , 1 g/L ammonium acetate, 0.2 g/L sodium citrate) with sterilized distilled H_2O and agar (1.5% w/v). The media were supplemented with sterile solutions of glucose (0.2% w/v final), $MgSO_4 \cdot 7H_2O$ (1 mM final). For propagation of the bacteriophage PRD1 and the spot test with PRD1 the LB broth with 1.5% NaCl (w/v) was used.

All media were supplemented, if required, with Millipore filter-sterilized amino acids, vitamins and antibiotics at the following concentrations: amino acids, 40 $\mu g/mL$; vitamin B1, 5 $\mu g/mL$; ampicillin 100 $\mu g/mL$ for *E. coli* strains containing plasmids and 1000 $\mu g/mL$ for *Klebsiella* strains carrying plasmids; chloramphenicol, 100 $\mu g/mL$; tetracycline, 20 $\mu g/mL$; kanamycin, 50 $\mu g/mL$; streptomycin, 50 $\mu g/mL$, spectinomycin, 50 $\mu g/mL$ for *E. coli* strains, or 150 $\mu g/mL$ for *Klebsiella* strains; rifampicin, 100 $\mu g/mL$; nalidixic acid, 25 $\mu g/mL$. The antibiotics were purchased from Sigma Chemical Co. and were prepared as stock solutions by dissolving appropriate concentrations in solvent (H_2O for most, or ethanol for

chloramphenicol and tetracycline, 1N NaOH for nalidixic acid) and stored at 4°C. Rifampicin was prepared fresh in methanol.

Restriction enzymes and linkers were purchased from New England BioLabs Ltd., Mississauga, Ontario. T4 DNA ligase, Klenow enzyme and 1 kb MW standard were purchased from GIBCO-BRL Life Technologies Ltd., Burlington, Ontario. *Taq* DNA polymerase was purchased from Promega. 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was purchased from Boehringer-Mannheim Canada Ltd. [32 P]-Orthophosphoric acid (specific activity 9120 Ci/mmol), 5'-triphosphate tetra(triethylammonium) salt, [α - 32 P] (specific activity 3000 Ci/mol) and [35 S]-L-methionine (specific activity 710 Ci/mmol) were purchased from Amersham Canada Ltd., Oakville, Ontario. [14 C(U)]-L-proline (specific activity 260 mCi/mmol) and [14 C]-methyl- α -D-glucopyranoside (specific activity 275 mCi/mmol) were obtained from DuPont Canada. Primers for PCR and sequencing were made using a model 391 PCR-MATE EPTM DNA synthesizer (Applied Biosystems) and are listed in Table 3. Other fine chemicals were from Sigma Chemical Corp., U.S.A.

Bacterial growth conditions.

All strains were grown in LB in a shaker overnight at 37°C. Exponential cultures were prepared by diluting the overnight culture 100 fold or from a single colony and then regrowing them to give a viable cell count of $1-2 \times 10^8$ cell/mL. Stock cultures were maintained at -20°C in LB mixed 1:1 with 80% (v/v) glycerol in TE (pH 8.0).

Table 1. List of bacterial strains used.

Strain	Genotype/Phenotype	Source or Reference
<i>Escherichia coli</i> K-12		
AB2847	<i>aroB thi malT tsx</i>	Braun, 1989
BR158	AB2847 <i>tonB</i>	Braun, 1989
BL21(DE3)	<i>hsdS gal (λclts857 ind1 Sam7 nin5 lacUV5-T7 gene1)</i>	Studier and Moffatt, 1986
C600	F ⁻ <i>thi thr leuB6 lacY1 tonA21 supE44 λ:</i>	Bachmann, 1972
C600Nal ^R	Same as C600 but resistant to 60μg/mL nalidixic acid	This laboratory
CC118	<i>sup^o (ara leu)7697 lacx74 phoA20 galE galK thi rpsE rpoB argE am recA1</i>	Manoil and Beckwith, 1985
DH5α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 λ·φ80 dlacZ M15 Δ(lacZYA-argF)U169</i>	Jessee, 1986
<i>Klebsiella oxytoca</i>		
UN2979	<i>hisD4226 lacZ4001</i> galactose resistant	MacNeil <i>et al.</i> , 1981
<i>Salmonella typhimurium</i>		
TA100	<i>hisG46 rfa ΔuvrB</i>	Ames <i>et al.</i> , 1975

Table 2. List of plasmids used.

Plasmid	Marker(s)	Description	Source or Reference
pCU1	Ap,Sm,Sp	parental plasmid	Konarska-Kozłowska and Iyer, 1981
pCU1 <i>lacZ</i>	Ap,Sm,Sp Km	Tn <i>phoA'</i> -1 in pCU1	This study
pCU57Δ14	Cm, Tc	pCU1 coordinates 3.9 - 9.55 in pACYC184	Paterson and Iyer, 1992
pCU66	Ap, Sm, Km	Tn5 in pCU1	Thatte <i>et al.</i> , 1985b
pCU403	Km	deletion derivative of pCU66	Thatte <i>et al.</i> , 1985a
pCU403-1	Km	<i>KpnI</i> deletion of pCU403	Rodríguez de González, 1994
pCU56	Cm, Tc	<i>kikB-oriT BglII</i> fragment of pCU1 in pACYC184	Thatte <i>et al.</i> , 1985a
pCU56Kan	Km, Tc	Km ^R derivative of pCU56	This study
pCU801	Ap,Sm,Sp	<i>oriT</i> derivative of pCU1	Rotheim <i>et al.</i> , 1988
pVT149-1	Cm	<i>oriT</i> derivative of pCU1	Rodríguez de González, 1994
pKM101	Ap	deletion of R46	McCann <i>et al.</i> , 1975
pACYC184	Cm, Tc	cloning vector	Chang and Cohen, 1978

Table 2. continued

pBR322	Ap, Tc	cloning vector	Bolivar <i>et al.</i> , 1977
pET23d	Ap	expression vector	Rosenberg <i>et al.</i> , 1987
pJF118HE	Ap	expression vector	Fürste <i>et al.</i> , 1986
pPR111	Ap	expression vector	Windle, 1986
pPR111Ω	Sm,Sp	expression vector	This study
pUC1318	Ap	cloning vector	Kay and McPherson, 1987
pUC1813	Ap	cloning vector	Kay and McPherson, 1987
pNEB193	Ap	cloning vector	New England Biolabs
pKK232-8	Ap	promoter-probe vector	Brosius, 1984
pUC1318kikA	Ap	<i>kikA</i> in pUC1318	This study
pUC1813kikA	Ap	<i>kikA</i> in pUC1813	This study
pPH0/1	Cm	<i>kikA</i> in pACYC184	Hengen, 1991
pPH4	Ap	<i>kikA</i> in pJF118HE	Hengen, 1991
pMH5	Sm,Sp	<i>kikA</i> in pPR111Ω	This study
pMH70	Ap	<i>orf70</i> in pJF118HE	This study
pMH70Ω	Sm,Sp	Ω (Sm/Sp ^R) derivative of pMH70	This study
pMH70-T7	Ap	<i>orf70</i> in pET23d	This study
pMH104	Ap	<i>orf104</i> in pJF118HE	This study
pMH104-T7	Ap	<i>orf104</i> in pET23d	This study
pNEB-P ₁	Ap	promoter P ₁ in pNEB193	This study

Table 2. continued

pNEB-P₂	Ap	promoter P₂ in pNEB193	This study
pNEB-P₁₋₂	Ap	promoters P₁₋₂ in pNEB193	This study
pKK-P₁	Ap	promoter P₁ in pKK232-8	This study
pKK-P₂	Ap	promoter P₂ in pKK232-8	This study
pKK-P₁₋₂	Ap	promoters P₁₋₂ in pKK232-8	This study
pAAG8	Ap	pCU1 coordinates 30 to 32.2 in pCU128	Rodriguez de González, 1994
pCU56Kan-G8	Km	pCU1 coordinates 30 to 32.2 in pCU56Kan	This study
pHP45Ω	Sm/Sp	source of Sm^R/Sp^R Ω fragment	Prentki and Krisch, 1984

Table 3. List of oligonucleotide primers used for PCR and/or nucleotide sequencing.

Designation	DNA sequence	Source
104	5'-ATCGGTGGACCATGGATTAAAAGGAG ATATACATATGAAGAACTCTTAATA-3'	Queen's University
104/1	5'-CGATGAATTCTATAAGCGAACTTTCCC GTATTT-3'	"
ORF70/1	5'-ACTGACATGTTGAAGATCCCTGCAAAG-3'	Carleton University
ORFI	5'-CTGCCAGCCACGCTGAAGATCCCTGCA-3'	"
ORFII	5'-TTCAGCGTGGCTGGCAGGAAGATAA-3'	"
MH1	5'-ATCGAATTCGAGGTTTGTGTCGAAT-3'	"
MH2	5'-ATCGGATCCAATGTTGATAAAGGTAAG-3'	"
MH3	5'-ATCGAATTCAATCCATTTATAAGGCAGCGTTA-3'	"
MH4	5'-ATCGGATCCTTTGTTGATTTTTTGTCTT-3'	"

Bacterial matings.

Bacterial matings were performed for determination of plasmid transfer or for the construction of plasmid-carrying strains. An overnight culture of the plasmid-carrying donor strain grown in LB containing the appropriate antibiotic(s) was diluted 100 fold in fresh media and grown to a cell density of $1-2 \times 10^8$ cell/mL. 1 mL of the donor culture was mixed with 1 mL of a stationary recipient culture and the mixture was collected on a Millipore membrane (47 mm, 0.45 μ m pore size). The membrane was placed on a prewarmed LB plate and incubated for 1 hour at 37°C. Mating was interrupted by vortexing the membrane in 1 mL of saline (0.85% w/v NaCl). Serial dilutions were prepared in saline and were plated on selective media for transconjugants. The plates were incubated overnight at 37°C.

Plasmid pCU1 killing of *Klebsiella*.

A qualitative test for the ability of donor cells to kill *Klebsiella oxytoca* was performed by growing donor cells to mid-exponential phase and spotting 5 μ L onto a lawn of recipient cells (grown to stationary phase) on Davis minimal media supplemented with glucose and histidine. Plates were incubated overnight at 37°C. A clear zone in the region where the donor was spotted was an indication of killing.

Phage infection test.

100 μ L of the exponentially-grown cells harbouring the appropriate plasmid were spread on a prewarmed modified LB plate (1.5% NaCl) and 10 μ L of bacteriophage PRD1 stock (3×10^{12} pfu/mL) was spotted on the plate. Plates were incubated overnight at 37°C.

Radioactive labelling of the bacteriophage PRD1 *in vivo*.

Bacteriophage DNA labelling and the purification of the labelled phage were performed essentially as described by Kotilainen *et al.* (1993) with modifications. *Salmonella typhimurium* strain TA100 carrying the plasmid pKM101 was grown overnight in 20 mL of minimal medium and 100 μ L of bacteriophage PRD1 (10^{11} pfu/mL) was added. The culture was left at room temperature for 10 minutes and a 300 μ Ci of [32 P]-orthophosphoric acid (Amersham) was added. The culture was then incubated for 3 hours at 37°C on a rotatory shaker. Cells were pelleted by centrifugation for 10 minutes at 6,000 $\times g$ and the phage particles were precipitated from the supernatant by adding solid polyethyleneglycol PEG-8000 (10% w/v final) and solid NaCl (0.5 M final). The mixture was incubated on ice for 2 hours and the phage particles were pelleted by centrifugation for 10 minutes at 10,000 $\times g$. The pellet was resuspended in 1 mL of buffer (0.01M phosphate buffer, pH 7.2; 1 mM $MgCl_2$) and filtered through a 0.45 μ m Millipore filter. The phage titer was obtained by plating phage dilutions on the *S. typhimurium* TA100 (pKM101). The radioactivity of the sample was measured by suspending 5 μ L of the labelled phage in 5 mL of ScintiVerse SX18-4 scintillation liquid and measuring the radioactivity using the Beckman LS 7500 Liquid Scintillation System. The specific activity obtained was 8.96×10^4 cpm/pfu.

Phage adsorption assay.

(i) **Phage binding.** *E. coli* C600 cells carrying the appropriate plasmid were grown to 1×10^9 cell/mL and mixed with the radiolabelled phage at a multiplicity of infection (MOI) of 0.1. After 15 minutes of incubation at 37°C the cells were separated by centrifugation for

5 minutes in the Eppendorf centrifuge and washed twice in 0.5 mL saline (0.85% w/v NaCl) to remove non-specifically bound phage. The final pellet was resuspended to the original volume of saline and the radioactivity of the samples was determined using a scintillation counter.

(ii) Localization of phage DNA. *E. coli* C600 cells carrying the appropriate plasmid were grown to 1×10^9 cell/mL and mixed with the radiolabelled phage at a MOI of 0.1. After 25 minutes of incubation at 37°C, the cells were separated by centrifugation for 5 minutes in the Eppendorf centrifuge and washed twice in saline (0.85% w/v NaCl) to remove non-specifically bound phage. The final pellet was resuspended in the periplasmic buffer (40 mM TrisCl, pH 8.0, 4 mM EDTA, 0.5 M sucrose)(Wood, 1978) and lysozyme was added to a final concentration of 1 mg/ml. After 2 minutes of incubation at room temperature, $MgCl_2$ was added to a final concentration of 10 mM and cells were incubated for an additional 15 minutes at 37°C. Spheroplasts were separated by centrifugation and the radioactivity was measured in both the supernatant (periplasm + cell surface) and the pellet (spheroplasts).

Extraction of plasmid DNA.

1. Small scale preparation of plasmid DNA.

Two methods were used for rapid extraction of plasmid DNA. The modified alkaline lysis method of Birnboim and Doly (1979) was used to extract plasmid DNA for the rapid analysis of recombinants. *E. coli* cells with appropriate plasmids were grown in 3 mL overnight culture in LB broth in the presence of appropriate antibiotics. The cells were pelleted in the Eppendorf centrifuge for 5 minutes and the pellet was resuspended in 100 μ L

of chilled Solution I (50 mM glucose, 25 mM TrisCl (pH 8.0), 10 mM EDTA (pH 8.0)). 200 μ L of lysis solution (0.2 M NaOH; 1% w/v SDS) was added to the cell suspension and mixed by inversion. The samples were incubated at room temperature for 5 minutes until lysis was complete. 150 μ L of neutralization solution (3 M potassium acetate, pH 4.8) was then added and the content was mixed quickly by inversion. The samples were incubated at -20°C for 20 minutes and were then spun in an Eppendorf centrifuge (approximately 14,000 $\times$ g) for 15 minutes at 4°C. The clear supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of phenol. After a brief spin in the Eppendorf centrifuge, the upper aqueous layer was transferred to a fresh Eppendorf tube and extracted with chloroform : isoamylalcohol (24:1). The aqueous phase was again separated by brief centrifugation and was transferred into a fresh Eppendorf tube. $\frac{1}{2}$ volume of 10 M ammonium acetate was then added and the DNA was precipitated by adding 2-3 volumes of ice-cold absolute ethanol and placing the tubes at -20°C for 30 to 60 minutes. Precipitated plasmid DNA was recovered by spinning in an Eppendorf centrifuge for 30 minutes at 4°C and the DNA pellet was washed with 70% (v/v) ice-cold ethanol. The DNA pellet was then dried in a vacuum dry oven at 50°C for 10 minutes and dissolved in 20 μ L of TE buffer (10 mM TrisCl, pH 8.0; 1 mM EDTA, pH 8.0).

For purposes of DNA sequencing and PCR mutagenesis a MagicMiniprep™ purification system (Promega) was used. 3 mL of the overnight culture was pelleted in the Eppendorf centrifuge for 5 minutes and the pellet was resuspended in 200 μ L of the cell resuspension solution (50 mM TrisCl, pH 7.5; 10 mM EDTA; 100 μ g/mL RNase A). 200 μ L of the cell lysis solution (0.2 M NaOH; 1% w/v SDS) was added and the sample was mixed

by inversion. The sample became clear and viscous almost immediately and 200 μ L of the neutralization solution (2.0 M potassium acetate, pH 4.8) was added. The sample was gently mixed by inversion and spun immediately in the Eppendorf centrifuge for 6 minutes at 4°C. Clear supernatant containing plasmid DNA was mixed with 1 mL of MagicMiniprep™ purification resin and mixed by inversion. The mixture was placed into a 3cc disposable syringe with attached MagicMiniprep™ purification column and pushed slowly through. DNA bound to the resin became immobilized on the column and was then washed with 2 mL of the MagicMiniprep™ washing solution (100 mM NaCl; 10 mM TrisCl, pH 7.5; 2.5 mM EDTA; 50% v/v ethanol). The column was detached from the syringe and spun in the Eppendorf centrifuge for 20 seconds to remove any wash solution that had been left over. DNA was eluted from the column by adding 50 μ L of distilled sterile H₂O (68°C), incubating for 1 minute and spinning for 20 seconds in the Eppendorf centrifuge.

2. Large scale preparation of plasmid DNA.

Large amounts of plasmid DNA were isolated by the method of Clewell and Helinski (1969) with the modification described by Humphreys *et al.* (1975). 1000 mL of an overnight culture of the desired strain grown in the presence of antibiotic was pelleted by centrifugation at 5,000 xg for 10 minutes at 4°C in a GSA rotor. The cells were resuspended in 30 mL of 25% sucrose (w/v), 50 mM TrisCl (pH 8.0) and divided into two SS34 tubes. 3 mL of 250 mM EDTA (pH 8.0) was added (per tube) and the mixture was incubated on ice for 5 minutes, then 1 mL (per tube) of fresh lysozyme (10 mg/mL in 250 mM EDTA, pH 8.0) was added and the tubes were mixed by gentle inversion. Next, 4 mL (per tube) of Triton-lysis

solution (25 mM EDTA, 50 mM TrisCl, pH 8.0, 2% v/v Triton X-100) was added and the tubes were incubated at 37°C for 5-10 minutes, mixing occasionally until lysis occurred. When the sample became viscous it was centrifuged at 4°C for 45 minutes at 30,000 xg using the SS34 rotor; this step pelleted the cell debris and most of the chromosomal DNA while plasmid DNA was recovered in the supernatant. To a clear supernatant, $\frac{1}{3}$ volume of 40% w/v PEG-6000 in 2M NaCl was added, the sample was mixed gently by inversion and left at 4°C overnight. The precipitate that was formed was pelleted by centrifugation using a SS34 rotor at 8,000 xg for 10 minutes and was dissolved in 1.5 mL of TES buffer (10mM TrisCl, pH 8.0, 1 mM EDTA, 100 mM NaCl). At this point, both samples were combined into a new SS34 tube. 0.8 mL of ethidium bromide (4 mg/mL stock solution in TE buffer) and 4 g of solid CsCl were added and dissolved. The refractive index was determined by using a Bausch and Lomb refractometer and was adjusted to 1.390 ± 0.002 using CsCl-saturated TE buffer. The sample was centrifuged in a Beckman VTi 80 rotor using a Beckman L8-80 ultracentrifuge at 305,000 xg for 4 hours. After centrifugation, the plasmid DNA band was recovered by puncturing the tube with a 3 cc disposable hypodermic syringe and withdrawing the plasmid band under the illumination of a long wave ultraviolet lamp.

Ethidium bromide was extracted from the DNA solution by adding CsCl-saturated isopropanol and discarding the alcohol layer which contained the ethidium bromide. This step was repeated several times until all visible traces of the ethidium bromide were removed. The plasmid DNA solution was dialysed against TE buffer (pH 8.0) at 4°C overnight and was then precipitated by adding 2 volumes of ice-cold ethanol, $\frac{1}{6}$ volume of 10M ammonium acetate and overnight incubation at 4°C. The DNA was pelleted by centrifugation in a HBX rotor at

10,000 xg for 10 minutes. The precipitated DNA pellet was dried and dissolved in distilled H₂O. The approximate concentration of DNA in the sample was determined by measuring the optical density at 260 nm of a known dilution of the sample. An O.D. of 1.0 is equal to 50 µg/mL of double-stranded DNA (Maniatis *et al.*, 1982).

Restriction endonuclease digestion of DNA.

The digestion of plasmid DNA was performed with 1 µg of DNA in a total volume of 20 µL. The reaction mixture contained 2 µL of the 10x reaction buffer (as recommended by the supplier), 2 µL of BSA solution (10 µg/mL), sterile distilled water and restriction enzyme (1-2 units per 1 µg of DNA). The digest was incubated for one hour at the recommended temperature for the specific enzyme. When using the DNA extracted from mini-preps, the digestion was performed in the presence of RNase solution (0.5 mg/mL). If the digestion was to be run on an agarose gel, $\frac{1}{10}$ volume of the loading buffer (0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol; 40% w/v sucrose in water) was added prior to electrophoresis.

Analysis of DNA by gel electrophoresis.

Plasmid DNA cut with restriction enzymes was separated by agarose gel electrophoresis in 0.7% to 1.5% agarose gel in TBE buffer (10.8 g Tris base, 5.5 g boric acid, 4 mL 0.5M EDTA (pH 8.0)). The gel was run in a horizontal, submerged, mini cell electrophoretic unit at 8 V/cm. The current for electrophoresis was supplied by an EPS power supply (Pharmacia Fine Chemicals). To separate small DNA fragments polyacrylamide gel

electrophoresis (PAGE) was performed. The DNA was run on a 1 mm thin 6% polyacrylamide gel (acrylamide : bisacrylamide ratio 50:1) in TBE buffer in the BioRad vertical electrophoretic cell unit at 10 V/cm. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/mL) and destained in distilled water. Gels were illuminated by a Fotodyne Foto/prep I transilluminator and photographed using a Polaroid MP4 vertical camera using a red filter with type 57 or 55 Polaroid film. The DNA standard used was BRL 1 kb DNA ladder with fragment sizes of: 12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 517; 506; 396; 344; 298; 220; 201; 154; 136 and 75 base pairs (bp). The computer program DNAFrag (Schaffer and Sederoff, 1981) was used to calculate the fragment sizes of digested DNA.

Recovery of DNA from the gel.

The restriction fragments from either purified or mini-prep DNA were recovered from the electrophoretic gels for subcloning. The agarose gel fragment containing the band of interest was sliced out of the gel using a razor blade and transferred into a pouch made from Parafilm which was then placed into -20°C for 5 to 10 minutes. The gel piece was then squeezed thoroughly and was placed into -20°C for 5 to 10 minutes again. After a thorough squeezing the liquidified agarose containing DNA was transferred into an Eppendorf tube and the Parafilm "pouch" was washed with 100 µL of TE buffer (pH 8.0). The DNA was extracted twice with phenol and once with chloroform : isoamylalcohol (~4:1). $\frac{1}{5}$ volume of 10 M ammonium acetate was then added to the DNA containing aqueous phase. DNA was precipitated by adding 2-3 volumes of ice-cold absolute ethanol and 1 µg of tRNA and

placing the tubes at -20°C for 1 to 2 hours. Precipitated DNA was pelleted by spinning in an Eppendorf centrifuge at 4°C for 30 minutes. The DNA pellet was washed in ice-cold 70% v/v ethanol, dried in a vacuum dry oven at 50°C for 10 minutes and resuspended in 10 µL of sterile distilled water.

Restriction fragments separated by PAGE were isolated in a similar fashion. The DNA fragment of interest was excised from the gel by slicing with a razor blade and transferred to an empty Poly-Prep Chromatography column (BioRad). The slice was then ground with a siliconized glass rod, overlaid with 350 µL of elution solution (0.5M ammonium acetate; 0.1% w/v SDS; 1 mM EDTA; 10% v/v methanol) and was soaked overnight at room temperature. The column was then spun in a clinical table top centrifuge for 10 minutes at $\frac{3}{4}$ speed and washed with 100 µL of elution solution. 450 µL of eluent was then extracted twice with 500 µL of phenol and once with 500 µL of chloroform : isoamylalcohol (24:1). The DNA containing aqueous phase was transferred to an Eppendorf tube and the DNA was precipitated by adding 3 volumes of ice-cold absolute ethanol and incubating at -20°C for 1 to 2 hours or in the liquid nitrogen for 2x5 minutes. Precipitated DNA was pelleted by spinning in an Eppendorf centrifuge at 4°C for 30 minutes. The DNA pellet was washed in ice-cold 70% v/v ethanol, dried in a vacuum dry oven at 50°C for 10 minutes and resuspended in 10 µL of sterile distilled water.

Ligation of DNA.

Ligations of DNA were done by combining DNA fragments with vector molecules in a 10 µL reaction using T4 DNA Ligase (1 unit per reaction; 2 units per reaction with blunt

ends) (GIBCO-BRL) and 5x T4 DNA Ligase buffer supplied by the manufacturer. The ligation mixtures were incubated at 15°C overnight and were then used to transform the appropriate competent cells. For blunt end ligations, blunt ends were prepared by treating free DNA ends with the Klenow enzyme (GIBCO-BRL) using the mixture of four dNTPs in Klenow enzyme buffer supplied by the manufacturer. The fill-in reaction was stopped by extracting the DNA with phenol and chloroform as described above.

Transformation of bacterial cells.

Transformation of bacterial cells was performed by a procedure similar to that of Cohen *et. al.* (1972). A single colony was inoculated into LB broth containing the appropriate antibiotic and grown at 37°C to an optical density of 0.3 - 0.4. The cells were then chilled on ice for 5 to 10 minutes and pelleted using the SS34 rotor at 5,000 xg for 10 minutes at 4°C. The cells were resuspended in $\frac{1}{6}$ of the original volume of ice-cold 0.1 M CaCl_2 and were kept on ice for 1 to 2 hours. The cells were again centrifuged as before and resuspended in $\frac{1}{20}$ of the original volume of ice-cold 0.1 M CaCl_2 and were kept on ice overnight. 100 μL of competent cells was used for transformation with 50-200 ng of plasmid DNA. The transformation mixture was placed on ice for 30 minutes. The cells were then heat pulsed at 42°C for 2 minutes and placed back on ice for 1 minute. 1 mL of fresh LB broth was then added and the cells were incubated at 37°C for 2-4 hours before plating on selective media.

Southern blot transfer and DNA hybridization conditions.

For the purpose of identification of positive clones in some clonings, the DNA was transferred onto a solid matrix by a modified version of the technique of Grunstein and Hogness (1975) as described in Maniatis *et al.* (1982). The bacterial colonies were "lifted" onto a Hybond-N nylon membrane (Amersham Canada, Ltd.) by placing it over a LB plate with grown recombinant colonies. The membrane was then placed (colony side up) on a 3MM Whatman paper soaked in 10% w/v SDS. After 3 minutes of incubation, the membrane was placed on a 3MM Whatman paper soaked in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 5 minutes and then the membrane was placed on a 3MM Whatman paper soaked in neutralization solution (0.5 M TrisCl; 1.5 M NaCl; 1 mM EDTA; pH 7.2) for 5 minutes. After this treatment the membrane was placed on a dried sheet of membrane paper and was air-dried for 60 minutes. The DNA was bound to the membrane by inverting the membrane on a UV transilluminator for 2 minutes.

A radiolabelled probe was prepared by restriction of purified DNA and recovery of the fragment from the gel. Labelling of the fragment was by nick translation (Rigby *et al.*, 1977) as described by Maniatis *et al.* (1982) with approximately 0.1 to 0.5 µg of DNA and 25 µCi of radiolabelled [α -³²P]-dATP (Amersham Canada, Ltd.) using the nick translation kit purchased from Bethesda Research Labs, Inc. (BRL). The labelled DNA was recovered by precipitation with 300 µL of ice-cold absolute ethanol, 10 µL of 10M ammonium acetate and 1 µg of tRNA. The tube was placed into liquid nitrogen for 10 minutes and the DNA was pelleted by centrifugation in the Eppendorf centrifuge for 15 minutes at maximum speed. The DNA pellet was washed in 70% v/v ice-cold ethanol and resuspended in 20 µL of sterile

distilled water. The probe DNA was denatured by boiling for 5 minutes prior to adding it to a hybridization tube. Hybridization was done by a modified protocol supplied by Amersham. Prehybridization was done in a hybridization incubator (Model 310, Robbins Scientific) at 65°C for 3 to 4 hours in a prehybridization solution (5x SSC; 10x Denhardt's solution; 0.5% w/v SDS; 250 µg/ml of denatured salmon sperm DNA (Sigma) and 10% w/v dextran sulphate). The prehybridization solution was then replaced by 1 mL of hybridization solution (5x SSC; 10x Denhardt's solution; 0.5% w/v SDS; 250 µg/ml of denatured salmon sperm DNA (Sigma)) containing the denatured radiolabelled probe and the hybridization was performed overnight at 65°C. After hybridization the membrane was washed twice with 50 mL of 1x SSC, 0.1% w/v SDS for 20 minutes at 65°C and then twice in 50 mL of 0.1x SSC, 0.1% w/v SDS for 20 minutes at 65°C. After the washes, the membrane was air-dried and placed inside a cassette with a DuPont intensifying screen and exposed to Kodak-X-OMAT AR film at room temperature overnight. The film was developed using an AFP Imaging 14XL X-ray film processor with chemicals for processing purchased from Picker Chemicals.

DNA Sequencing.

DNA sequencing was done by using Sequenase version 2.0 provided in a sequencing kit purchased from United States Biochemical Corporation (USB), Cleveland, Ohio, U.S.A. The method for sequencing was the dideoxy-termination technique described by Sanger *et al.* (1977). The ssDNA template was prepared by denaturing 2-4 µg of plasmid DNA in 0.2 M NaOH for 5 minutes. The reaction was stopped by adding $\frac{1}{3}$ volume of 5 M ammonium acetate and 4 volumes of ice-cold absolute ethanol and placing the mixture into liquid

nitrogen for 5 minutes. DNA was pelleted by centrifugation in the Eppendorf centrifuge for 10 minutes, washed in 70% v/v ice-cold ethanol and dried in the vacuum oven. The sequencing reaction was done by annealing 0.5 pmol of the synthetic oligonucleotide primer to the ssDNA template at 65°C for 2 minutes and slowly cooling it to 30°C. The primer was extended with radiolabelled deoxyadenosine [α -³²P]-triphosphate (Amersham) for 5 minutes at room temperature as recommended by the supplier of the Sequenase sequencing kit. The dideoxy-termination step was carried out at 38°C for 4 minutes and the reactions were terminated by adding the stop solution provided with the kit. Each reaction was denatured by boiling for 5 minutes before loading 2-3 μ L onto a 6% sequencing polyacrylamide gel (acrylamide:bisacrylamide ratio 19:1; 42% w/v urea) and electrophoresed at 28 mA for the desired length of time. After electrophoresis, the gel was transferred to a 3MM Whatman paper, covered with SaranWrap and dried in a gel dryer. The gel was then placed on Kodak X-Omat AR film, exposed overnight at room temperature and developed as described above.

Computer assisted DNA and protein sequence analysis.

Data base searches for DNA and amino acid sequences were done by using the NCBI BLAST network (blast@ncbi.nlm.nih.gov) (Altschul *et al.*, 1990), FASTA (fasta@ebi.ac.uk) (Pearson and Lipman, 1988) and PROSITE (prosite@embl-heidelberg.de) (Bairoch, 1993). Prediction of the signal sequence was done by the method of von Heijne (1986) using the Unix shareware program SigSeq, and the prediction of the secondary protein structure was done by a PredictProtein program via a EMBL network server in Heidelberg (predictprotein@embl-heidelberg.de) (Sander and Schneider, 1991). Analysis of nucleotide

sequences was performed using a MacDNASIS Pro. V3.0 (Hitachi Software Engineering Co. 1993).

PCR-directed mutagenesis and PCR cloning.

PCR cloning and PCR-directed mutagenesis was performed essentially as described by Higuchi (1990) by using *Taq* DNA polymerase (Promega). PCR reactions were done in 25 μ L volumes using the following conditions: 1x reaction buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 15-20 pmol of each primer, 0.1-0.5 μ g DNA template and 2.5 units of *Taq* DNA polymerase (Promega). For PCR cloning the DNA was amplified using a Hybaid Combi Thermal reactor TR2 with the following cycle: 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 25 repetitions the cycle was changed to 94°C for 1 minute, 55°C for 1 minute and 72°C for 5 minutes (1 cycle) and the reactions were terminated by placing tubes at -20°C. 6 μ L of the amplified DNA was cut with the appropriate restriction enzyme and run on a 6% polyacrylamide gel. The DNA fragment of interest was isolated and cloned as described above.

The PCR-directed mutagenesis is schematically shown in Figure 2. The primary PCR reactions were done using the same conditions as for PCR cloning. However, due to the mismatch between the primer and the template, the thermal cycle was modified as follows: first 5 cycles at 94°C for 40 seconds; 52°C for 40 seconds; 72°C for 40 seconds. Next 20 cycles were performed at 94°C for 40 seconds; 55°C for 40 seconds; 72°C for 40 seconds. The last cycle was at 94°C for 40 seconds, 55°C for 40 seconds and 72°C for 5 minutes. 10 μ L of the amplified DNA was run on the 6% polyacrylamide gel and the DNA fragment

Figure 2. A schematic drawing of the PCR cloning (Higuchi, 1990). The primers used for PCR are indicated in small characters. Two new restriction sites are indicated as RS1 and RS2, a mutation introduced by PCR-directed mutagenesis is shown as X. The DNA template is indicated as a solid black line, and the PCR products are indicated as interrupted lines.

Primary PCR
PAGE Electrophoresis
Isolation of DNA Fragments

Secondary PCR

Cloning

isolated as described above. A secondary PCR reaction was performed with equal amounts of "right" and "left" PCR products under the same conditions as above with a thermal cycle as follows: 12 cycles at 94°C for 1 minute; 55°C for 1 minute; 72°C for 1 minute, and the last cycle was at 94°C for 1 minute; 55°C for 1 minute and 72°C for 5 minutes. 6 µL of the amplified DNA was cut with the appropriate restriction enzyme, run on a 6% polyacrylamide gel, isolated and cloned as described above.

Gel electrophoresis of proteins.

3 mL of *E. coli* DH5- α , BL21 or *K. oxytoca* cells carrying the appropriate plasmid were grown in LB to O.D.₅₅₀ ~0.6 and induced with 0.5 mM IPTG for 3 hours. Cells were separated by centrifugation and resuspended in 100 µL 1x protein loading buffer (2% w/v SDS; 5% v/v 2- β -mercaptoethanol; 10% w/v sucrose; 0.002% w/v bromophenol blue). The samples were boiled for 5 minutes and then spun in the Eppendorf centrifuge for 1 minute. The supernatant was transferred into a fresh Eppendorf tube and was stored at -20°C. 1-2 µL of protein samples were run on 17.5% SDS-PAGE (4.5% stacking gel) using the Tris-glycine discontinuous buffer system described by Hames (1981). After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 (0.1% w/v in water:methanol:glacial acetic acid (5:5:2)) for 1-2 hours at room temperature and destained extensively in 12.5% v/v isopropanol, 10% v/v acetic acid. The gels were preserved in BioGel Wrap™ Gel Drying System (BioDesign Inc.).

Due to the small size of the expected proteins a second type of protein electrophoresis, described by Anderson *et al.* (1983), was also used. Protein samples were

prepared essentially as above but the proteins were solubilized in 0.139 M Tris base, 0.5% w/v SDS and 20% w/v sucrose (pH 7.8 with glacial acetic acid). The samples were applied on a 12% SDS-PAGE gel (8 M urea) and 5 μ L of 2% w/v Pyronin Y, 50% w/v sucrose, 0.01% w/v sodium azide was injected into each well. After electrophoresis the gel was stained, destained and preserved as described above. Since this gel electrophoresis system does not incorporate any reducing agents (β -mercaptoethanol or DTT) the mobilities of the samples are different when compared to the system of Hames.

Isolation of the periplasmic proteins and partial protein purification.

The technique of Wood (1978) was used to isolate the periplasmic proteins. 3 mL of *E. coli* DH5- α , BL21 or *K. oxytoca* cells carrying the appropriate plasmid were grown in LB to O.D.₅₅₀ ~0.6 and induced with 0.5 mM IPTG for 3 hours. Cells were separated by centrifugation and resuspended in 50 μ L of periplasmic buffer (0.5 M sucrose; 4 mM EDTA; 40 mM TrisCl, pH 8.0; 0.25 mg/mL lysozyme). After 2 minutes of incubation at room temperature 1 M MgCl₂ was added to a final concentration of 10 mM and the samples were incubated at 30°C for 30 minutes. Spheroplasts were separated from the periplasmic proteins by centrifugation in the Eppendorf centrifuge for 20 minutes at 4°C. The supernatant was removed and mixed with equal volumes of 2x protein loading buffer and 5-20 μ L of the sample was run on the SDS-PAGE gel as described above.

The periplasmic fraction of the Orf104 protein was further precipitated with 80% (w/v) ammonium sulphate for 2 hours at 0°C to salt out contaminating proteins and most of the lysozyme. Precipitated proteins were pelleted in the Eppendorf centrifuge for 10 minutes

at 4°C and the purified protein sample (supernatant) was dialysed against 10 mM TrisCl (pH 7.2), 10 mM EDTA and was stored at -20°C.

Protein sequencing.

The periplasmic protein fraction was purified on a 15% SDS-PAGE gel and was electroblotted onto a Problot PVDF membrane (Applied Biosystems). After staining with Coomassie Blue R250, the correct band was sliced out with a razor blade and the N-terminal part of the protein was sequenced on a 475A protein sequencing system incorporating a 470A Applied Biosystems gas phase sequencer and 120A PTH amino acid analyzer under the control of a model 900A data analysis module (this work was kindly performed by David Watson, NRC Canada).

Mass spectrometer analysis of the Orf104 protein.

The partially purified protein sample (0.1 mg/mL) was extensively dialysed against 5% acetic acid (2x24 hours) and was then analyzed on the VG Quattro Triplequadropole Mass Spectrometer (Fisons Instruments) with a flow rate of 4 µL/min (this work was kindly performed by David Watson, NRC Canada).

Measurement of DNA, RNA and protein synthesis.

Synthesis of DNA, RNA and proteins was determined by measuring the incorporation of [³²P] and [³⁵S]-L-methionine into TCA precipitable material essentially as described by Watson (1972). An overnight culture of *Klebsiella oxytoca* carrying the plasmid pMH5 was

diluted in fresh LB broth supplemented with the appropriate antibiotics and grown at 30°C to O.D.₅₅₀ ~ 0.8. The bacterial culture was then divided into two parallel samples diluted in fresh LB broth to O.D.₅₅₀ ~ 0.05 and grown at 30°C for 1 hour on the shaker. At that time [³²P] H₃PO₄ (2.5 µCi/mL) and [³⁵S]-L-methionine (6µCi/mL) were added simultaneously and samples were incubated for 15 minutes at 30°C on the shaker (prelabelling, t= -30 min.). After an additional 30 minutes of incubation at 30°C, one parallel sample was transferred to 42°C (t=0). 50 µL samples (6x) were withdrawn in 5 minute interval (from t=-30 to t=180 min.) and pipetted onto 3MM Whatman filter disks and air dried for 1 minute. Two disks from each time point were then immersed in 10% (w/v) TCA at 4°C for 30 minutes. The disks were then washed three times with 5% (w/v) cold TCA and three times with a 1:1 ether-ethanol mixture in a Buchner funnel. The disks were then dried in an oven at 50°C for 45 minutes, placed into scintillation vials containing 5 mL of ScintiVerse SX18-4 scintillation fluid and counted using a Beckman LS 7500 Liquid Scintillation System. This treatment is referred to as the cold TCA method and the results obtained represent the incorporation of [³²P] into total nucleic acids (both DNA and RNA). Two other disks of each time point were treated as follows. The disks were immersed in 1M NaOH and incubated at 37°C overnight. The NaOH was then replaced with ice cold 10% (w/v) TCA, the disks were chilled on ice for 30 minutes and treated using the cold TCA method as described above. Radioactivity remaining after this alkali treatment is that incorporated into DNA. These values were subtracted from the cold TCA precipitable counts to give a value for the incorporation of [³²P] label into RNA. The remaining two disks were treated by the hot TCA method to determine the incorporation of [³⁵S]-L-methionine into the cellular proteins. The disks were incubated in 10% (w/v) TCA on

ice for 30 minutes and then in 5% TCA (w/v) at 85°C for 30 minutes. The disks were then washed in the Buchner funnel and processed as described above for the cold TCA method.

Measurement of the uptake of macromolecules.

An overnight culture of *Klebsiella oxytoca* carrying the plasmid pMH5 (*kikA*) or the plasmid pPR111Ω (control) was diluted in fresh LB broth supplemented with the appropriate antibiotics and grown at 30°C to O.D.₅₅₀ ~ 0.1. The samples were then shifted to 42°C and were incubated for an additional 3 hours while shaking. At that time the samples were washed twice in saline (0.85% w/v NaCl) and resuspended in 10 mL of Davis minimal medium. The O.D.₅₅₀ of the samples was then adjusted to ~0.5 by adding Davis minimal medium. Samples were incubated for 15 minutes at 42°C at which time the radioactive label was added ([³⁵S]-L-methionine (2 μM, 0.6μCi/mL), [¹⁴C] methyl-α-D glucopyranoside (α-MG, 20 μM, 0.5 μCi/mL)). Samples (100 μL) were withdrawn in 30 second intervals for 5 to 10 minutes and pipetted onto 0.45 μm filters secured in a vacuum manifold. Filters were immediately washed with 5 mL of saline and transferred into scintillation vials containing 5 ml of ScintiVerse SX18-4 scintillation fluid and were counted in a Beckman LS 7500 Liquid Scintillation System. The relative rate of uptake (% of control) was calculated for the first 2 minutes of uptake and was calculated from two independent measurements. The uptake of L-proline and the effect of KCN on the uptake of L-proline was performed under the same conditions with the exception that plasmids pPH4 (*kikA*) and pJF118HE (control) were used instead of the plasmids pMH5 and pPR111Ω. Induction of *kikA* was achieved by incubation with 1 mM IPTG for 3 hours. After the final resuspension of the cells in the Davis minimal medium, cells

were preincubated with 0.01 M KCN for 2 minutes and then glucose (0.2 % (w/v)) was added. The radioactive L-proline ($[^{14}\text{C}]$ -L-proline (2 μM , 0.05 $\mu\text{Ci/mL}$)) was added after 5 minutes and samples were taken and processed as described above. Control cells were treated with saline instead of KCN. (The uptake of α -MG was performed under the kind supervision and in the laboratory of Dr. D. Sprott, NRC Canada. Other experiments were performed, using the same method, at Carleton University)

Retention of proline.

An overnight culture of *Klebsiella oxytoca* carrying the plasmid pPH4 (*kikA*) or the plasmid pJF118HE (control) was diluted in fresh LB broth supplemented with the appropriate antibiotics and grown at 37°C to O.D.₅₅₀ ~ 0.1. At that time 1 mM IPTG was added and the cells were incubated for an additional 3 hours at 37°C. The cells were washed twice in saline (0.85% w/v NaCl) and resuspended in Davis minimal medium to O.D.₅₅₀ ~ 0.5. $[^{14}\text{C}]$ -L-proline (2 μM , 0.05 $\mu\text{Ci/mL}$) was added and the cells were incubated for 10 minutes at 37°C. A 1 mL sample was withdrawn and pipetted onto 0.45 μm filter and washed with 5 mL of saline immediately. The remaining cell suspension was centrifuged for 5 minutes in a bench-top centrifuge, washed once with saline and resuspended in the original volume of Davis minimal medium. The cell suspension was incubated at 37°C and samples (1 mL) were taken in 1 minute intervals, pipetted onto 0.45 μm filter secured in a vacuum manifold and washed immediately with 5 mL of saline. Filters were then placed into scintillation vials containing 5 mL of ScintiVerse SX18-4 scintillation fluid and counted in a Beckman LS 7500 Liquid Scintillation System. Values were corrected for the loss of radioactivity during centrifugation.

Determination of bacterial respiration.

An overnight culture of *Klebsiella oxytoca* carrying the plasmid pPH4 (*kikA*) or the plasmid pJF118HE (control) was diluted in fresh LB broth supplemented with the appropriate antibiotics and grown at 37°C to O.D.₅₅₀ ~ 0.1. At that time 1 mM IPTG was added and the cells were incubated for an additional 3 hours at 37°C. The cells were washed twice in saline (0.85% w/v NaCl) and resuspended in PBS buffer (136 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄) (pH 7.4) to O.D.₅₅₀ ~ 0.9. Oxygen consumption was measured using an Orion Research Model 97-08 oxygen electrode connected to an Orion 601A digital ionoanalyzer. Readings were taken in 5 second intervals. The cellular respiration was induced by adding filter-sterilized glucose (20%) to a final concentration of 0.4%. Inhibition of respiration (KCN, 0.01 M final) was used to confirm that the observed consumption of oxygen was due to respiration.

Isolation of total cellular RNA, gel electrophoresis and Northern hybridization.

Total cellular RNA was isolated using the TRIzol™ Reagent (Gibco BRL) using the protocol provided by the manufacturer. An overnight culture of *Klebsiella oxytoca* carrying the appropriate plasmids was diluted in fresh LB broth supplemented with the appropriate antibiotics and grown at 37°C to O.D.₅₅₀ ~ 0.4. At that time 10 mM IPTG was added and the cells were incubated for an additional 3 hours at 37°C. 1.5 mL of the cell suspension was pelleted by centrifugation in an Eppendorf centrifuge (approximately 14,000 xg) for 3 minutes at 4°C. The pellet was resuspended in 1 mL of TRIzol reagent and the cells were lysed by

repetitive pipetting. Homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of the nucleoprotein complexes. 0.2 mL of chloroform was then added and the samples were shaken vigorously by hand for 15 seconds and then incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000 xg for 15 minutes at 4°C in the Eppendorf centrifuge. Following centrifugation, the mixture separated into a lower red, phenol-chlorophorm phase, an interphase, and a colourless upper aqueous phase containing RNA. The upper aqueous phase was carefully transferred to a fresh tube. RNA was precipitated with 0.5 mL of isopropanol. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 xg for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 1 mL of 75% ethanol (DEPC-treated). Samples were mixed by vortexing and centrifuged at 7,000 xg for 5 minutes at 4°C. At the end of the procedure the RNA pellet was vacuum-dried for 10 minutes and dissolved in 10 µL of DEPC-H₂O by passing the solution through a pipette tip a few times and incubating for 10 minutes at 60°C.

To prevent contamination with RNases, the equipment used for the work with RNA was carefully treated with the RNase inhibitor DEPC (diethylpyrocarbonate) and all solutions were prepared in DEPC-treated water. DEPC-treated water was prepared by adding 0.1% (v/v) DEPC to distilled H₂O, incubating at 37°C overnight and autoclaving. The electrophoretic tank and glassware used were rinsed with soap water, absolute ethanol and were air dried. They were then soaked for 20 minutes in 3% (v/v) H₂O₂ and rinsed extensively with DEPC-treated water. Gel electrophoresis of RNA was performed as described by Mariatis *et al.* (1982). The RNA was separated using a 1.4% (w/v) agarose gel supplemented

with formalin (2.2 M final) which was run in the RNA running buffer (0.1 M MOPS (pH = 7.8), 5 mM DEPC-EDTA (pH = 8.0), 40 mM DEPC-sodium acetate). Prior to the loading of the RNA samples, 4.5 μ L of the total RNA was mixed with 3.5 μ L of formalin, 10 μ L of formamide and 2 μ L of 5x RNA running buffer. The samples were incubated at 65°C for 15 minutes and then chilled on ice. 2 μ L of the RNA loading buffer (50% (v/v) glycerol, 1mM DEPC-EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol in DEPC-water) was added to each sample as well as 1 μ L of EtBr (1mg/mL in DEPC-water). The gel was prerun for 10 minutes at 5 V/cm prior to the loading of the samples. The samples were run at 3 - 5 V/cm. After electrophoresis, the gels were illuminated by a Fotodyne Foto/prep I transilluminator and photographed using a Polaroid MP4 vertical camera using a red filter with type 57 Polaroid film.

For the purpose of the identification of the *kikA* RNA transcripts, the RNA was transferred onto a Hybond-N nylon membrane as described by Southern (1975), with some modifications. The RNA gel was rinsed extensively (2 x 30 minutes) in DEPC-water and was then neutralized in neutralization solution (0.5 M Tris-Cl; 1.5 M NaCl; 1 mM EDTA, pH 7.2) for 2 x 30 minutes. It was then placed on a glass sheet and it was covered with a piece of Hybond-N nylon membrane, one piece of 3MM Whatman filter paper saturated with 3xSSC buffer (0.45 M NaCl, 45 mM sodium citrate) and a stack (about 5 cm) of paper towels followed by a light weight. The transfer was allowed to occur overnight, after which time the filter was removed and washed gently in 2x SSC (0.3 M NaCl, 30mM sodium citrate) in order to remove any traces of agarose. The filter was air dried and the RNA was bound to the nylon membrane by exposure to UV light for 2 minutes.

The labelling of the 500 bp *kikA* region which was used as a probe was performed exactly as described above (Southern transfer and DNA hybridization conditions, p.51 of this section). The pre-hybridization and hybridization conditions used were the same as well. After hybridization, the membrane was washed once in 1xSSC, 0.1 % w/v SDS for 20 minutes at room temperature and then three times in 0.2xSSC, 0.1 % w/v SDS for 20 minutes at 68°C. After the washes, the membrane was air dried and placed inside a cassette with a DuPont intensifying screen and exposed to Kodak-X-OMAT AR film at room temperature overnight. The film was developed using an AFP Imaging 14XL X-ray film processor with chemicals for processing purchased from Picker Chemicals.

Construction of the plasmid pCU1*lacZ*.

An overnight culture of CC118(pCU1) grown in LB broth was infected with the λ Pam phage carrying Tn*phoA*'-1 transposon element at MOI of 0.1 essentially as described by Wilmes-Reisenberg and Wanner (1992). After overnight incubation at 37°C the cells were spread on LB plates supplemented with Km, Sm, Sp, and Ap and incubated overnight. Colonies which appeared on the plates the next day were pooled and mated with *E. coli* C600Nal using standard filter mating. Transconjugants carrying the putative pCU1-Tn*phoA*'-1 construct were then screened for the Kik⁺ phenotype by spot test as described on page 40 of this section. DNA from one Kik⁺ clone was extracted and the position of the Tn*phoA*'-1 was mapped by restriction analysis.

Determination of the levels of expression from the *kikA* putative promoters.

Putative promoters of *kikA* were cloned into a promoter-probe vector pKK232-8 as described in the Results and Discussion section, Part 2. Plasmid pKK232-8 is sensitive to chloramphenicol and the insertion of a promoter into this plasmid will result in the acquisition of resistance to chloramphenicol. *E. coli* DH5 α and *Klebsiella* UN2979 cells carrying plasmid derivatives of interest were grown overnight in LB broth supplemented with appropriate antibiotics and diluted 1:100 into fresh LB broth supplemented with appropriate antibiotics. The samples were then grown to O.D.₅₅₀ 0.9 - 1.0. 5 μ L of each sample was then spotted onto a series of prewarmed LB plates supplemented with chloramphenicol (1 μ g/mL to 400 μ g/mL, increment by 2.5 μ g/mL). The plates were incubated overnight at 37°C and scored for the presence or absence of growth. As a negative control, *E. coli* DH5 α and *Klebsiella* without any plasmid were tested using the same test. It was found that both *E. coli* and *Klebsiella* did not grow on 2 μ g/mL of chloramphenicol.

Results and Discussion.

Part 1.

The killing of *Klebsiella* by plasmid pCU1 is not post-segregational.

Naturally occurring plasmids of the incompatibility group N of *Escherichia coli* K-12 are generally conjugative and are known to have a broad host range which includes *Klebsiella* species (Iyer, 1989). It has been observed, however, that introduction of an IncN group plasmid into at least some *Klebsiella* reduces their viability (Rodríguez, 1981; Rodríguez and Iyer, 1981). Under similar conditions of mating, *Escherichia coli* recipients do not display a reduction in viability thus identifying the phenomenon as host-specific. Killing is not mediated by any substance which diffuses from *E. coli* to *Klebsiella* and, with *E. coli* carrying the wild type plasmid, requires plasmid transfer by bacterial mating (Rodríguez and Iyer, 1981).

Recently, a number of plasmids of Gram negative bacteria have been shown to possess systems which enable them to kill, selectively, plasmid-free segregants that arise in the population (see Literature Review for details). This part of the thesis will attempt to answer the question whether the Kik⁺ phenotype mediated by plasmid pCU1 can have a similar basis with the qualification that it occurs, selectively or preferentially, in *Klebsiella*.

1.1 Construction of the pCU1*lacZ* derivative.

Since the plasmid pCU1 is lethal to the *Klebsiella* host, selection for transconjugants following bacterial matings can result in the recipients being killed by or inhibited either by the plasmid or by the antibiotics used in selection. It would be more informative if one could follow the fate of the plasmid in the population of *Klebsiella* recipients by screening rather than by selecting for transconjugants. To do this, a derivative of pCU1 carrying the Tn5-based transposable element Tn*phoA'*-1 (Wilmes-Reisenberg and Wanner, 1992) was constructed as described in the Materials and Methods section. In the Tn*phoA'*-1 element *phoA* is replaced

by *lacZ*. This element also carries a kanamycin-resistance marker. The transposon vector was delivered on λ Pam (Wilmes-Reisenberg and Wanner, 1992) and the derivatives of pCU1 were screened for the presence of the *Tra*⁺ and *Kik*⁺ phenotypes and for the retention of plasmid-borne antibiotic-resistance markers (*Ap*^r, *Sp*^r, *Sm*^r). One such derivative called pCU1*lacZ* was isolated and the position of *TnphoA*'-1 on it was mapped, by restriction analysis, to the plasmid coordinate 16.5 counterclockwise to its replication region (not shown). The plasmid was then introduced into *E. coli* strain C600 by mating and was maintained in this strain.

1.2 Bacterial matings with C600(pCU1*lacZ*).

C600(pCU1*lacZ*) was mated with the *Klebsiella* UN2979 and, separately, with *E. coli* DH5 α strains as outlined in the Materials and Methods section. Dilutions of each mating mixture were spread on minimal agar supplemented with histidine (requirement for UN2979) and thiamine (requirement for DH5 α) and X-gal. The results are summarized in Table 1.1. As seen in this table, transconjugant colonies of *E. coli* were uniformly blue as would be expected for a population that receives and maintains the plasmid in a stable manner. Furthermore, their numbers were approximately the same whether they were screened as *Lac*⁺ colonies or selected as *Km*^r colonies (Table 1.1). These colonies, when tested, were found to have retained all the plasmid-borne and the transposon-borne markers. When the *Klebsiella* were used as a recipient there was a striking difference. When transconjugants were screened on X-gal, their numbers (as determined by colonies showing evidence of *Lac* expression) were much greater than when transconjugants were selected as *Km*^r colonies (Table 1.1). Furthermore, while the colonies arising at low frequency on the medium with *Km* were

Table 1.1. Frequency and type of transconjugants in the matings with an auxotrophic *E. coli* donor (C600) carrying pCU1*lacZ* and *E. coli* or *Klebsiella* as recipient.

Recipient	Fraction (%) of survivors on Minimal Agar ^a		
	total	on X-gal Blue ^b	on X-gal + Km
<i>E. coli</i> DH5 α	88.37	23.25	24.65 ^c
<i>K. oxytoca</i> UN2979	2.59	0.31	0.0015 ^c

^a the fractions indicated are in relation to a control population of the recipient treated in a manner similar to the mating experiment but without the donor. The Minimal Agar was supplemented with thiamine (for DH5 α) or with histidine (for UN2979).

^b as determined by colonies showing any evidence of Lac expression, i.e. completely blue colonies in the case of *E. coli* recipients or predominantly white colonies with blue areas in the case of *K. oxytoca* recipients as in Figure 1.1.

^c uniformly blue colonies

uniformly blue, those arising on the media without Km were for the most part white but with a small and irregular blue region (Figure 1.1). Cells from the white and blue areas of these colonies were purified to homogeneity and then tested for the presence of the antibiotic markers. None (100 tested) of the clones from the white region carried any of the plasmid-borne (Sm^R, Sp^R, Ap^R) including the transposon-borne (Km^R) antibiotic markers. These clones were therefore inferred to be plasmid-free. Clones purified from the blue regions were now uniformly blue and they carried all plasmid- and transposon-borne antibiotic resistance markers.

Figure 1.2 is an interpretation of these observations in the context of the Kik' phenotype conferred by pCU1. The Kik' phenotype is evident in *Klebsiella* but not in *E. coli*. After the plasmid is received by *Klebsiella* (Figure 1.2a) the expression of Kik arrests the growth of the recipient (Figure 1.2b). However, if the cell is capable of division and loses the plasmid, it will resume growth, resulting in the majority of the colony being white (Figure 1.2c to e). The blue areas observed on the white colonies are neither sectors nor outgrowths but discrete areas close to the surface or edge of the colonies (Figure 1.1). These areas arise when the plasmid persists in the "mother cell" for a period that is long enough for a rare secondary chromosomal mutation to occur (Figure 1.2f,g). This mutation confers immunity to the Kik' phenotype and occurs late during the development of a colony. An alternative possibility that the chromosomal mutation occurs in a cell that constitutes the plasmid-free part of the colony (white area) and that such a cell subsequently accepts pCU1/lacZ from the rare "mother cell" that has not yet died is unlikely because in reconstruction experiments (not shown) plasmid transfer within the colony was not observed. If the chromosomal mutation does not occur

(and the cell does not lose the plasmid) it fails to form a colony and dies. This results in the reduction of the observed viability of *Klebsiella* recipients in the matings with pCU1.

This result is consistent with results shown in Table 1.1 and with those obtained by Rodriguez de González (1994). If *Klebsiella* transconjugants are selected for a pCU1-borne antibiotic resistance markers, the frequency of transconjugants is at least a thousand-fold less than if transconjugants are identified by screening on the X-gal plates. This is due to the fact that in the former situation the recipients are constrained to maintain the plasmid. They are able to do this by acquiring a chromosomal mutation which confers immunity to the Kik⁺ phenotype of pCU1.

The patterns of inheritance of the plasmid pCU1*lacZ* in *Klebsiella* populations presented here indicate clearly that the plasmid is deleterious to the host when present in the cell and that loss of the plasmid is not deleterious. The Kik⁺ phenotype is therefore not a consequence of segregational plasmid loss followed by the killing of plasmid-free cells.

Figure 1.1. Appearance of colonies of *Klebsiella oxytoca* UN2979 cells that have received pCU1*lacZ* and lost the plasmid (white areas of colonies) or retained it in the few cells in the colony that have acquired a chromosomal mutation (blue areas of colonies).

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Figure 1.2. Diagrammatic interpretation of the consequences of introducing a Kik⁺ plasmid pCU1lacZ into *Klebsiella* and the emergence of sub-population of plasmid-free but non-immune (majority) and plasmid-carrying but immune (minority) cells within a colony.

Following the introduction of a Kik⁺ plasmid into a *Klebsiella* cell (a, b), expression of the Kik⁺ phenotype can have one of three outcomes. There can be a segregational loss of the plasmid (c) allowing the cell to survive and multiply (d, e) to give rise to a large number of plasmid-free cells in the colony. A second possibility is that the presence of the plasmid inhibits the multiplication of the cell so that the plasmid is only unilinearly inherited (broken arrow). During such inheritance, the cell can either irreversibly lose colony-forming ability (shown as X) or survive by acquiring, secondarily, a chromosomal mutation (f) that confers immunity to Kik⁺ (shown as I). This secondary mutation arises only late in the development of the clone so that immune *Klebsiella* constitute a minority of the colony (g).

Part 2.

Physiological consequences of the expression of *kikA*.

The conclusion from the first section of Results and Discussion was that the Kik⁺ phenotype required the continued presence of pCU1 in *Klebsiella* and did not arise from the post-segregational loss of pCU1. Hengen (1991) showed that when this 500 bp region of pCU1 alone was cloned and overexpressed in *Klebsiella*, this was sufficient to reduce the viability of the host. The physiological consequences of the expression of *kikA* in *Klebsiella* are examined in this section.

2.1. Cloning of the *kikA* region under the control of the λ_{P_R} promoter.

Hengen's experiments (1991) showed that the induction of *kikA* expression affects the viability of *Klebsiella* cells but not that of *E. coli*. It was also confirmed during this research that the viability and the growth rate of *E. coli* carrying the plasmid pPH4 was not affected by the addition of IPTG (results not shown). However, it was not clear whether this observed phenotype was due to the irreversible killing of the cells or reversible inhibition of their growth. In order to distinguish between these two possibilities the *kikA* region was recloned into a more suitable expression system which allows the change from permissive to non-permissive conditions by alteration of the incubation temperature.

A 500 bp *EcoRI-SphI* fragment of the plasmid pPH4 (Hengen, 1991) containing the *kikA* region was placed under the control of the λ_{P_R} promoter in the expression vector pPR111 Ω (Windle, 1986, modified)(Figure 2.1). The *EcoRI-SphI* fragment from pPH4 was first cloned into the cloning vector pUC1813 (Kay and McPherson, 1987) and then recloned as a *HindIII* fragment into the cloning vector pUC1318 (Kay and McPherson, 1987). Finally, the *BamHI* fragment containing the *kikA* region was cloned into the expression vector

pPR111 Ω creating the plasmid pMH5. The proper orientation of the fragment (allowing transcription of *orf104* and *orf70*) was confirmed by *HindIII* restriction analysis (not shown). The vector pPR111 Ω was itself created by inserting a Sm^R/Sp^R resistance Ω fragment (Prentki and Krisch, 1984) into a *PstI* site of the Ap^R gene of the plasmid pPR111. The resulting plasmid is now resistant to streptomycin and spectinomycin. In this way, Ap^R was replaced with Sm^R/Sp^R. This was desirable as plasmid-free *Klebsiella oxytoca* already displays significant resistance to the former antibiotic. Expression from the λP_R promoter is controlled by the thermosensitive repressor gene *cl857* located counterclockwise to the promoter. Expression of any gene cloned behind this promoter can be regulated by changing the temperature from 28 - 30°C (permissive) to 37 - 42°C (non-permissive). If *kikA* has a bacteriostatic effect on *Klebsiella* cells, then a temperature shift from 30°C to 42°C should inhibit the growth of the culture, while the shift back to permissive conditions will allow resumption of the growth. On the other hand, if *kikA* has a bactericidal effect, the damage after incubation at the non-permissive temperature should be permanent. The advantage of using this expression system over the IPTG inducible construct pPH4 used by Hengen (1991) is that the non-permissive condition can be more efficiently and rapidly changed back to the permissive condition. The resulting plasmid, pMH5, was transformed into *Klebsiella* strain UN2979 and the viability of the host under the permissive and non-permissive conditions was measured.

Figure 2.1. Construction of the plasmid pMH5 to place the 500 bp *kikA* region of pPH4 under the control of the λP_R promoter of the plasmid pPR111 Ω . The *SphI-EcoRI* fragment of pPH4 containing *kikA* was cloned into the plasmid pUC1813, yielding the plasmid pUC1813-*kikA*. The *HindIII* fragment of pUC1813-*kikA* was then recloned into the plasmid pUC1318, yielding the plasmid pUC1318-*kikA*. Finally, the *BamHI* fragment of pUC1318-*kikA* was inserted into the expression vector pPR111 Ω , yielding the plasmid pMH5. Arrows within circles indicate the direction of transcription and abbreviations within the circles are for the relevant promoters or genes. Abbreviations outside the circles are for restriction sites. *lacIⁿ* is a constitutively expressed gene encoding the *lac* repressor, *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon, cI857 is gene encoding thermo-sensitive λ repressor.

2.2. Expression of *kikA* halts cellular growth reversibly.

When *Klebsiella* cells carrying pMH5 were transferred from 30°C to 42°C, an inhibition of growth was observed (Figure 2.2). This arrest of growth is not immediate but has a lag period of 30 to 60 minutes. When the culture from the non-permissive temperature (42°C) was shifted back to the permissive temperature (30°C), it was found that cell growth continued after a lag period of 3 to 5 hours (Figure 2.3). These results indicate that:

- (i) the effect of *kikA* expression on the viability of *Klebsiella* cells is reversible,
- (ii) there is a lag period of about two cell divisions before the inhibitory effect can be observed, and
- (iii) the half life of the Orf104 and/or Orf70 proteins of *kikA* may be no more than 5 hours, since after the temperature shift into permissive conditions there is a lag period of 3 to 5 hours before cell growth is fully restored.

Induced cells were also analyzed under the light microscope. However, there were no morphological changes or deviations in shape and/or size observed (filamentation, clumping, swelling etc.) when compared to control cells (not shown).

2.3. Effect of *kikA* expression on cellular metabolism.

2.3.1. Effect of *kikA* expression on DNA, RNA and protein synthesis.

Incorporation of [³²P] into DNA and RNA was measured in an attempt to understand the mechanism of *kikA* action. Nucleic acids were labelled with [³²P]-H₃PO₄ and the amount of incorporated label was determined by the cold TCA method (see Materials and Methods). The results of these experiments are summarized in Figure 2.4. Both the DNA and RNA

Figure 2.2. Growth of *Klebsiella oxytoca* cells carrying the plasmid pMH5(•) or pPR111Ω(▽) after the temperature shift from 30°C to 42°C. Overnight cultures were diluted 1:100 and grown for 2 hours at 30°C. The temperature was then raised to 42°C and the optical density (O.D.₅₅₀) was measured. Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

Figure 2.3. Growth of *Klebsiella oxytoca* cells carrying the plasmid pMH5(•) or pPR111Ω(▽) after the temperature shift from 42°C to 30°C. Overnight cultures were diluted 1:100 and grown for 2 hours at 42°C. The temperature was then lowered to 30°C and the optical density (O.D.₅₅₀) was measured. Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

synthesis were arrested at the same time, between 40 to 60 minutes after induction. This lag period coincides with the lag observed when the optical density of the cell culture was measured (Figure 2.2).

Protein synthesis was measured by the hot TCA method with [^{35}S]-L-methionine. As shown in Figure R2.4 the cessation of protein synthesis seems to occur slightly before the synthesis of DNA and RNA is stopped. This result implies that the primary target of *kikA* is the protein synthetic machinery of the cell. However, since the synthesis of proteins was measured using the [^{35}S]-L-methionine label, an alteration in the uptake of methionine due to the expression of *kikA* would also be reflected in the synthesis of the proteins. Therefore, the uptake of the radiolabelled L-methionine was measured.

2.3.2. Effect of *kikA* expression on the uptake of small molecules.

The uptake of [^{35}S]-L-methionine was measured in order to investigate whether an arrest in protein synthesis is due to the interference of the *kikA* proteins with the transport of the radioactive label. As shown in Figure 3.5, upon *kikA* expression the uptake of methionine gradually decreased and three hours after the induction of *kikA*, uptake was fully inhibited (4% of the control). It was therefore inferred that the observed inhibition of protein synthesis was due to the prevention of the uptake of the radioactive label. Thus *kikA* is not interfering directly with protein, DNA or RNA synthesis. This result, supported by the fact that *kikA* has a bacteriostatic rather than a bactericidal effect, suggested that the expression of *kikA* interferes with a more general cellular function such as (i) respiration, (ii) membrane function and/or (iii) general fuelling reaction(s). The possibility that *kikA* interferes with the

Figure 2.4. Effect of *kikA* expression on DNA, RNA and protein synthesis in *Klebsiella oxytoca*. Cells carrying *kikA* under the control of the λP_R promoter (plasmid pMH5; solid symbols) or a control plasmid (plasmid pPR111 Ω ; hollow symbols) were grown to O.D.₅₅₀=0.1 at 30°C and then the temperature was shifted to 42°C. Synthesis of DNA (●), RNA (■) and proteins (▲) was followed by measuring the incorporation of ³²P-H₃PO₄ and ³⁵S-methionine respectively. The inset shows an enlargement of the time interval from 20 to 80 minutes.

Figure 2.5. Effect of *kikA* expression on the uptake of ^{35}S -methionine in *Klebsiella oxytoca*.

Cells carrying *kikA* under the control of the λP_R promoter (plasmid pMH5) or a control plasmid (plasmid pPR111 Ω) were grown to O.D.₅₅₀=0.1 at 30°C and then induced at 42°C. The uptake of ^{35}S -methionine was measured as described in the Materials and Methods section. The solid line represents the linear regression line ($r = -0.893$). The rate of uptake was calculated as an average of the two experiments.

function of the membrane was examined by measuring the uptake of different solutes into the induced and non-induced cells.

There are several trans-membrane transport systems operating in bacterial cells. Although the majority of the work elucidating transport in gram-negative bacteria was done in *Escherichia coli*, it was assumed in this work that *Klebsiella oxytoca* does not differ substantially from *E. coli* in transport mechanisms.

Bacterial cells can accumulate substrates of interest by several different mechanisms, such as passive diffusion, facilitated diffusion, active transport and group translocation (Kaback, 1972). However, only active transport and group translocation require metabolic energy and they can accumulate substrates against a concentration gradient. Therefore, an examination of a cell's ability to transport a particular substrate can reveal the physiological conditions of the cell.

It is generally believed that energy-coupling mechanisms of solute transport can be best interpreted by Mitchell's chemiosmotic theory of transport (Simoni and Postma, 1975). According to this theory, the hydrogen and electron carriers of the respiratory chain are arranged in loops across the cytoplasmic membrane in such a way that electron flow via the chain results in the translocation of protons from one side of the membrane to the other (Figure 2.6). Since the membrane is virtually impermeable to protons, the result is the generation of a proton motive force composed of membrane potential and a pH gradient. The proton motive force is linked to the transport processes in such a way that solute movement is coupled to the movement of protons. Proton motive force is also directly involved in the synthesis of cellular ATP via a membrane-bound proton-translocating ATPase (Kaback,

1972). However, the proton translocation is reversible and ATP hydrolysis can also catalyze proton extrusion. Therefore, there is an energy circuit consisting of respiration, oxidative phosphorylation, solute transport and ATP synthesis/hydrolysis.

The kinetic analysis of amino acid transport in *E. coli* K12 cells revealed the presence of several distinct transport systems with some overlap between them (Piperno and Oxender, 1968). Based on the effect of osmotic shock, it was shown that two categories of transport systems exist in bacteria (Berger and Heppel, 1974). The shock-sensitive systems are associated with periplasmic binding proteins and have an obligatory requirement of phosphate bond energy. The shock-resistant permeases are not released by osmotic shock and do not require energy from ATP. The shock-resistant systems appear to be driven by an energy-rich membrane state which can be formed by either respiration or ATP hydrolysis. It has been shown by Kadner and Winkler (1975) that proline uptake is driven directly by the energized membrane state but that the transport of methionine is similar to that of other osmotic shock-sensitive systems in its direct utilization of phosphate bond energy. Therefore, it was believed in this study that the differences in the uptake of proline and methionine may shed light on the physiological state of the *kikA*-induced cells.

Glucose, on the other hand, is transported across a membrane by a mechanism of group translocation (Kaback, 1972), utilizing the phosphoenolpyruvate sugar phosphotransferase system (PEP-PTS). PEP-PTS sugar uptake and phosphorylation require the participation of a number of soluble and membrane-bound enzymes which catalyze the transfer of the phosphoryl moiety of phosphoenolpyruvate to the sugar substrate (Dills *et al.*, 1980). The cell depleted of phosphate bond energy or with a physically damaged membrane

Figure 2.6. Schematic drawing of energy transduction across a bacterial membrane.

is not capable of transporting PTS sugars. An analogue of glucose, α -methylglucoside (α -MG), can be used in order to examine the PEP-PTS system instead of glucose. α -MG is transported by the same transport system as glucose but once inside the cell it cannot be further metabolized and thus makes measurements more accurate (Hagihira *et al.*, 1963).

The uptake of [14 C]- α MG and [14 C]-proline, substrates with different energy requirements than methionine, was examined. As seen in Figures 2.7 and 2.8, the uptake of both the α -MG and the L-proline was reduced after the induction of *kikA*. The transport of α -MG was reduced to 11% of the control. Since the uptake of L-methionine (see above) was also found to be reduced, these findings imply that the energy status of the cells is very low. The transport of proline, however, was reduced to only about 50% of the control. Since proline uptake is driven only by the energized state of the membrane, it seemed plausible that the inhibition of proline uptake is due to an insufficient membrane potential. However, as mentioned above, respiring cells generate the membrane potential by passing the protons across the membrane. Therefore, the uptake of proline was also examined in the presence of the respiratory poison KCN (Figure 2.8). It was found that if respiration is prevented, the uptake of proline is dramatically reduced (<1% of the control). This result suggests that the membrane potential of *kikA*-induced cells is disrupted and that this disables the cells from accumulating the solutes and generating the energy required for growth. It also indicates that respiration *per se* is not effected by *kikA* expression. The possibility that the expression of *kikA* prevents cellular respiration was tested by measuring the consumption of dissolved oxygen using a Clark electrode. As shown in Figure 2.9, there is no difference in respiration between the induced cells and the control cells. This result may explain why the expression

Figure 2.7. Effect of *kikA* expression on the uptake of ^{14}C - α -MG in *Klebsiella oxytoca*. Cells carrying *kikA* under the control of the λP_R promoter (plasmid pMH5) or a control plasmid (plasmid pPR111 Ω) were grown to O.D.₅₅₀=0.1 at 30°C and then induced at 42°C. The uptake of ^{14}C - α -MG was measured as described in the Materials and Methods section. The solid line represents the linear regression line ($r = -0.919$). The rate of uptake was calculated as an average of the two experiments.

Figure 2.8. Effect of *kikA* expression and KCN on the uptake of ^{14}C -proline in *Klebsiella oxytoca*. Cells carrying *kikA* under the control of the *tac* promoter (plasmid pPH4) or a control plasmid (plasmid pJF118HE) were grown to $\text{O.D.}_{550}=0.1$ at 37°C and then induced with 1 mM IPTG for 3 hours. The uptake of ^{14}C -proline was measured as described in the Material and Methods section. 0.01 M KCN (final concentration) was added 10 minutes before the first sample was taken. Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

Figure 2.9. Effect of *kikA* expression on respiration of *Klebsiella oxytoca*. Cells carrying *kikA* under the control of the *tac* promoter (plasmid pPH4; solid line) or a control plasmid (plasmid pJF118HE; dotted line) were grown to O.D.₅₅₀ = 0.1 at 37°C, were induced for 3 hours with 1 mM IPTG and resuspended in PBS buffer (pH 7.4) at room temperature. Glucose driven oxygen consumption was measured using an Orion research model 97-08 oxygen electrode and an Orion 601A digital ionoanalyzer as described in the Materials and Methods section.

of *kikA* does not result in the death of the cells, but only in the inhibition of their growth. The *kikA* protein(s) cause the dissipation of the membrane potential without affecting respiration. Therefore, as long as the cells are capable of carrying on respiration, the minimal membrane potential which is generated will prevent them from dying. However, once the resources which can be used for respiration are used up, the depolarization of the membrane will probably lead to the physical damage of the membrane and eventually to cellular death.

The depolarization of the membrane occurs if the membrane permeability is altered and the ions (H^+ , K^+ , Na^+) which normally cannot diffuse across the membrane, are now free to equilibrate. The intriguing question is whether the *kikA* proteins form a "hole" in the membrane or act on some other membrane structure(s) to facilitate the abolition of the membrane potential. It will be described and discussed in the next section (p.108 to p.128) that neither the Orf104 nor the Orf70 proteins of *kikA* has the features of a channel forming protein (membrane-spanning hydrophobic domains). While the Orf70 protein is located in the cytoplasm and is therefore unlikely to be involved in the channel structure, the Orf104 protein is periplasmic and it may be a Ca^{2+} -binding protein. It is tempting to speculate that the Orf104 protein can participate in the function of a pre-existing membrane channel and that the Orf70 protein can be a signal protein which can trigger opening and probably also a blockage of such a channel. The evidence that the Orf104 protein is involved in the translocation of the DNA across the cytoplasmic membrane will be presented in the Part 4. of this thesis and the model for the function of Orf104 and Orf70 proteins will be discussed in details.

The hypothesis of the *kikA* proteins not being channel proteins is also supported by

the fact that when the *kikA*-induced cells were preloaded with ^{14}C -proline, their ability to retain proline was the same as that of the control cells (Figure 2.10). Therefore, it can be concluded that the expression of *kikA*:

- (i) does not cause generalized cell leakiness,
- (ii) does not prevent respiration,
- (iii) reduces the uptake of methionine, proline and α -MG, which are substrates with different energy requirements, and
- (iv) if respiration is prevented, abolishes the uptake of proline completely.

Figure 2.10. Effect of *kikA* expression on the retention of ^{14}C -proline in *Klebsiella oxytoca*. Cells carrying *kikA* under the control of the *tac* promoter (plasmid pPH4, (•)) or a control plasmid (plasmid pJF118HE, (∇)) were grown to O.D.₅₅₀=0.1 at 37°C and then induced with 1 mM IPTG for three hours. Cells were pre-loaded with ^{14}C -proline for 10 minutes, washed and the retention of ^{14}C -proline was measured as described in the Materials and Methods section. The dotted lines represent the linear regression lines ($r = -0.1$ (pPH4); $r = -0.79$ (pJF118HE)). Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

Based on the results presented above the following model for the molecular mechanism of *kikA* action is proposed. Upon the expression of *kikA*, the channel is opened in the bacterial membrane. The opening of this channel will cause the ions to pass across the membrane along the concentration gradient resulting in the abolition of the membrane potential. This will probably also result in the reverse action of membrane bound ATPase which will start to hydrolyse ATP in order to restore the lost membrane potential. The final result is then the depletion of the energy. The cell is not capable of transporting solutes or of supporting growth. The only unaffected function is respiration, and the proton gradient generated by respiration prevents the cells from dying. However, if all the resources for respiration are used up, the depolarization of the membrane will eventually result in physical damage to the membrane and the ultimate death of the cells.

A similar mode of action was proposed for the channel forming colicins and some phages (Guihard *et al.*, 1993). It was noted that the phages and channel forming colicins promote a similar initial event but that the consequences for the host are different. Infection with some bacteriophages often leads to the efflux of ions across the membrane, hydrolysis of ATP and membrane depolarization. However, once the phage genome has been transferred, the cell membrane can repolarize and the cell is then capable of ATP synthesis and growth. This phenomenon is believed to be due to the closure of the membrane channel upon the transfer of the phage genome. On the other hand, the damage caused by channel forming colicins is permanent and leads to cell death. It was proposed that the channel formed by colicin oscillates between the open and the closed states as long as the respiratory chain is functional. However, the decrease in the internal concentration of potassium leads to an

inhibition of respiratory activity (Letellier, 1992). It is possible then, that the open/closed state of the colicin channel is only transient and will be eventually shifted towards the open state.

The results obtained with the *kikA*-induced cells suggest that *kikA* may represent a unique system with a resemblance to both the phage and the colicin induced phenomena. Unlike colicins, *kikA* acts from within the susceptible cell and the KikA proteins are not involved directly in the formation of the channel. However, upon expression of *kikA* there is a dissipation of the membrane potential which may eventually lead to cell death. Similar to the action of the phages, *kikA* seems to act on the already pre-existing channel in the membrane. Also, the product of one open reading frame of *kikA*, *orf104* (see Part 4 of Results and Discussion) is required for the infection by the bacteriophage PRD1.

Part 3.

Genetic analysis of the *kik1* region.

As discussed in the first part, the Kik⁺ phenotype conferred by pCU1 is due to the continuous presence of the plasmid in the *Klebsiella* host, and not to its segregational loss. Transposon and deletion mutagenesis of the plasmid pCU1 provided evidence that there are two independent regions, *kikA* and *kikB*, which are required for the Kik⁺ phenotype (Thattai *et al.*, 1985a). Hengen (1991) cloned and sequenced the *kikA* region, and showed that when the *kikA* region is placed under the control of an inducible promoter, the overexpression of *kikA* is sufficient to reduce the viability of *Klebsiella* (referred to as KikA⁺ in this thesis). It was therefore concluded that the *kikA* region plays a major role in the Kik⁺ phenotype. Results presented and discussed in the previous chapter suggested that the expression of *kikA* in *Klebsiella*, but not *E. coli*, leads to the induction of a membrane channel, dissipation of the membrane potential and energy depletion. The role of the *kikB* region in the Kik⁺ phenotype is indirect. Rodríguez de González (1994) showed that the absence of *kikB* leads to a structural instability of the plasmid in *Klebsiella*.

There are three open reading frames, *orf104*, *orf70* and *orf80*, in the 500 bp region of *kikA* (Figure 3.1)(Hengen, 1991). By cloning this region so that the *orf80* was under the control of the inducible *tac* promoter, Hengen (1991) showed that exposure of the *Klebsiella* cells to the inducer IPTG did not result in a KikA⁺ phenotype. On the other hand, similar induction of *Klebsiella* carrying the plasmid construct pPH4, in which *tac* could control transcription of both *orf104* and *orf70* did result in a KikA⁺ phenotype. Hengen's experiments thus suggested that the expression of both *orf104* and *orf70*, or the expression of one of them, may be sufficient for the KikA⁺ phenotype. His nucleotide sequence of the *kikA* region (Figure 3.2) also revealed that there are two putative promoters located upstream of *orf104*

Figure 3.1. Diagrammatic representation of the *kikA* region. The 500 bp region is shown as an open rectangle. Open reading frames are shown below the *kikA* region as arrows. The putative promoters P₁ and P₂, and the possible ribosome binding site (RBS), are shown as open boxes (drawn to scale). The numbers below the *kikA* region represent the scale in base pairs. Black triangle indicates position of *tac* promoter in plasmid pPH4.

Figure 3.2. The nucleotide sequence of *kikA* (Hengen, 1991). The start of each open reading frame is indicated with an arrow, translation termination sites are indicated with a (*). The putative promoters P₁ and P₂ are shown with the corresponding -10, -26 and -35 regions underlined. A possible ribosome binding site (RBS) is indicated by the open box.

AAGCTTGCATGCCTGCAGGTCGAGGTTTGTGTGCGAATATATTGCAGG

CGATATAGGTTACA⁻³⁵ATCCATT^{P₂}TATAACGCAGCGTT⁻¹⁰ATTATTAATATTACTT

ACCTTTATCAACATTCT⁻²⁶GGCGAGTGTAT^{P₁}⁻¹⁰CTGAACAACTGTTAAGGAC^{RBS}

AAAAATGAAGAACTCTTAATACCTCTGATAGCAGCTGGTAGTCTCCTT^{→ orf104}

^{↑ stop}
^{orf80}↓
TATCTTCCTGCCAGCCATGCTGAAGATCCCTGCAAAGTTATTATGTGCAT^{→ orf70}

GGCGGGCAAGCTCACCGGCGATAGCGGCGGAAGCGAGTGTAACAGTG

CTCAAGCTGCTTTCTTCAATATCGTTAAAAGAACAAGCACGGCTTTTACCC

AACCACACGAAGGATGCTAGGAAGGCTTTTCTTAATGAATGCCCCGGATAA

TGGCGAAGGTGGAAGTAACCAAGTCGAT^{↑ stop}
^{orf70}↓
GATAAGCCAGATCATAAGTAAAT^{orf80 ←}

ACGGGAAAGTTCGCTTAT^{↑ stop}
^{orf104}↓
AGGCCGGATCCCCGGGTACCGAGCTCGAATTC

and *orf70*, designated P₁ and P₂, respectively (Hengen, 1991).

In this chapter, the genetic analysis of the KikA⁺ phenotype and the analysis of the gene products of *kikA* are described and discussed.

3.1. The role of the individual *orfs* of *kikA* in the KikA⁺ phenotype: both *orfs* are needed for the KikA⁺ phenotype.

PCR-directed cloning and mutagenesis (Higuchi, 1990) was used to construct plasmid derivatives in which *orf104* and *orf70* of *kikA* were cloned separately under the control of artificially-introduced promoters.

The outside 5' oligonucleotide primer (104) used for the construction of plasmid pMH104 contained two restriction sites (*Sall*, *NcoI*) followed by a translation termination codon (TAA) and a ribosome binding site (AAGGAG) located 8 bp upstream of the ATG start codon. The outside 3' oligonucleotide primer (104/1) contained an *EcoRI* restriction site just downstream of a translation termination signal (see Table 3 in the Materials and Methods section for the list of primers used). In order to create a construct with the *orf104* only, the transition mutation T → C was introduced at nucleotide 230 using the inner primers ORFI and ORFII (Figure 3.3). Since *orf104* and *orf70* are not in the same reading frame, this mutation destroyed the start codon of *orf70* (ATG → ACG) but was neutral for *orf104* (CAT → CAC; His)(Figure 3.3). The *Sall* - *EcoRI* PCR product was cloned under the control of an inducible *tac* promoter in the expression vector pJF118HE, yielding the plasmid pMH104 (Figure 3.3). The *NcoI* - *EcoRI* restriction fragment of pMH104 containing *orf104* was further cloned into the expression vector pET23d (Rosenberg *et al.*, 1987), thus placing the *orf104* under the

Figure 3.3. Construction of the plasmid pMH104 carrying *orf104* under the control of an inducible *lac* promoter and of pMH104-T7 with *orf104* under the control of the T7 promoter. PCR cloning was used to mutagenize the start codon of *orf70* and to introduce restriction sites on 5' and 3' ends of the fragment. The 5' primer also contains translation termination codons and the ribosome binding site which were cloned in front of the ATG start codon of *orf104* (see Materials and Methods section). The primers used for PCR are shown in small, underlined numbers. The *Sall*-*EcoRI* PCR fragment was cloned into the expression vector pJF18HE, yielding the plasmid pMH104. The *NcoI* - *EcoRI* fragment of the plasmid pMH104 was then inserted into the expression vector pET23d, thus placing *orf104* under the control of the T7 promoter (plasmid pMH104-T7). The 500 bp *kikA* region is shown as an open rectangle at the top of the figure. The start of both open reading frames is shown with bent arrows. The asterisk denotes the mutated nucleotide (T → C'). This mutation destroys the start codon of *orf70* (ATG → ACG), but is neutral for *orf104* (CAT → CAC; His → His). *lacP* is a constitutively expressed gene encoding the *lac* repressor and *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon.

control of T7 polymerase promoter (Figure 3.3).

PCR cloning of *orf70* was performed with the 5' oligonucleotide primer ORF70/1 and the 3' oligonucleotide primer 104/1 (Figure 3.4). The primer ORF70/1 carries the base substitution C → T in the second codon of the *orf70* (nucleotide 232). Also, it introduced a dinucleotide AC in front of the ATG start codon of *orf70* (AC ATG). This substitution results in the neutral codon change CTTG → TTTG (Leu → Leu) and at the same time introduces an *AflIII* restriction site into the start codon of *orf70* (ATG CTTG → A'C ATG TTTG). This site was used to clone *orf70* into the *NcoI* site (C'CATGG) of the expression vector pET23d, which is located 8 bp downstream from the ribosome binding site, yielding the plasmid pMH70-T7 (Figure 3.4). This construct allows for the expression of *orf70* in the *E. coli* host BL21(DE3) which carries an IPTG inducible T7 RNA polymerase gene inserted into its chromosome. In a subsequent cloning, *orf70* was placed under the control of an inducible *tac* promoter in the expression vector pJF118HE, yielding the plasmid pMH70 (Figure 3.4). In this cloning, the *XbaI* - *EcoRI* restriction fragment of pMH70-T7 was ligated to *HindIII*(filled) - *EcoRI* sites of the plasmid pJF118HE. This fragment also carries a ribosome binding site from the vector pET23d which is located between the *XbaI* restriction site and the ATG start codon of *orf70*. Nucleotide sequencing was used to confirm the proper sequences of all plasmid constructs. The plasmid pMH70 was used for further cloning in which an ampicillin gene from the plasmid pMH70 was replaced with the Sm/Sp resistant Ω fragment (Prentki and Krisch, 1984), yielding the plasmid pMH70- Ω . The new construct pMH70- Ω can now be used *in trans* with plasmid pMH104. Even though these plasmids have the same replicon, different antibiotic markers allow for the selection of both plasmids

Figure 3.4. Construction of plasmids pMH70, pMH70 Ω and pMH70-T7 carrying *orf70* under the control of inducible *tac* and T7 promoters, respectively. PCR cloning was used to introduce restriction sites on the 5' and 3' ends of the fragment. The 5' primer (ORF70/1) has a base substitution C $\rightarrow$ T which introduces a new restriction site (*AflIII*) into the ATG start codon of *orf70* (see Materials and Methods section). This mutation does not affect the amino acid sequence of the Orf70 polypeptide (CTG $\rightarrow$ TTG; Leu $\rightarrow$ Leu). The primers used for PCR are shown in small, underlined numbers. The *AflIII*-*EcoRI* PCR fragment was cloned into the expression vector pET23d (*NcoI* - *EcoRI*), yielding the plasmid pMH70-T7. The *XbaI*-*EcoRI* fragment of pMH70-T7, containing *orf70* and the ribosome binding site of pET23d, was recloned into the *HindIII* (filled) and *EcoRI* sites of the plasmid pJF118HE, thus placing *orf70* under the control of the *tac* promoter (plasmid pMH70). The plasmid pMH70 was used in further cloning in which a Sm^R/Sp^R Ω fragment (Prentki and Krisch, 1984) was inserted into an Ap^R gene, yielding the plasmid pMH70 Ω . The 500 bp *likA* region is shown as an open rectangle at the top of the figure. The start of the open reading frame is shown with a bent arrow. *lacI^R* is a constitutively expressed gene encoding the *lac* repressor and *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon.

The plasmids pMH104, pMH70, pJF118HE (negative control) and pPH4 (positive control) were transformed into *Klebsiella* cells. Also, plasmid pMH70- Ω was transformed into *Klebsiella* cells already carrying the plasmid pMH104. The viability of the cells upon IPTG induction was measured and the results are summarized in Figure 3.5. As seen in this figure, expression of both *orfs in trans* or *in cis* is required for the inhibition of growth. Neither *orf104* nor *orf70* alone was able to inhibit cell growth. The combination of *orf104* and *orf70 in trans* seems to have a more profound effect on the viability of *Klebsiella* than the same combination *in cis*. This may be related to the fact that in the PCR constructs pMH104 and pMH70 transcription and transcripts of *orf104* and of *orf70* are independent of each other. It may be recalled (Figure 3.1) that in the corresponding region of the naturally-occurring plasmid there are putative promoters for each of these two open reading frames while in the plasmid pPH4 transcription of *orf104* and of *orf70* is driven only from one promoter (*tac*). The major conclusion from these experiments was that the presence of both *orf104* and *orf70* is essential for the KikA⁺ phenotype.

Figure 3.5. Viability of *Klebsiella oxytoca* cells carrying various combinations of *kikA* open reading frames *in cis* or *in trans* cloned under the control of an inducible *tac* promoter. An overnight culture was diluted 1:100 and grown in the presence of 0.5 mM IPTG at 37°C. The samples were withdrawn at one hour intervals and the O.D. (550 nm) was determined. Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

(■) p.F118HE (control)

(●) pPH4 (*orf104* and *orf70 in cis*)

(▼) pMH70Ω (*orf70* only)

(v) pMH104 (*orf104* only)

(□) pMH104 and pMH70Ω (*orf104* and *orf70 in trans*)

3.2. Isolation, partial purification and characterization of the Orf104 protein.

Klebsiella cells carrying the plasmid pMH104 were used to overproduce, partially purify and sequence the Orf104 protein. IPTG induced cells were treated with lysozyme to release periplasmic proteins (Wood, 1978), and the remaining spheroplasts were used to isolate cytoplasmic proteins. All samples were analyzed using 17.5% SDS-PAGE electrophoresis (Hames, 1981) and stained with Coomassie Brilliant Blue. Gel electrophoresis revealed that there is a water soluble protein located in the periplasm which is seen upon IPTG induction (Figure 3.6), and which is absent from the samples isolated from control cells. This protein band is therefore likely to be the Orf104 protein. The protein band of the same size was also observed in the samples isolated from *E. coli* DH5 α (results not shown). The molecular weight of this protein was calculated to be 8.85 kDa using a BioRad broad range standard. This estimated size is, however, much smaller than the size predicted from the DNA sequence of *orf104* (11,322 Da). Figure 3.7 shows the predicted amino acid sequence of the Orf104 protein, along with the predicted tertiary structure (Sander and Schneider, 1991). The computer program MacDNASIS was used to calculate some of the biophysical properties of the Orf104 protein, and these are summarized in Table 3.1. The isoelectric point (pI) was calculated to be 8.2, mean hydrophobicity was calculated to be -0.03 and the net charge was calculated to be +3. The hydrophobic profile of Orf104 (Kyte and Doolittle, 1982) is depicted in the Figure 3.9.

The fact that the Orf104 protein is found in the periplasm suggests that this protein is translocated across the cytoplasmic membrane. It is well documented for proteins translocated across biological membranes that these proteins contain an N-terminal

Figure 3.6. SDS-PAGE (17.5%) analysis of the protein samples isolated from the *Klebsiella oxytoca* cells carrying the plasmid pMH104 or pJF118HE (control). Cells were grown to O.D. (550 nm) ~ 0.5 and induced with 0.5 mM IPTG for 3 hours. Isolation of the periplasmic and cytoplasmic proteins was performed as outlined in the Materials and Methods section. The arrow indicates the Orf104 protein.

- 1. pJF118HE - periplasmic fraction**
- 2. pMH104 - periplasmic fraction**
- 3. MW standard (broad range, BioRad)**

Figure 3.7. Predicted amino acid sequence and the tertiary structure of the Orf104 protein.

The amino acid sequence, confirmed by microsequencing of the N-terminus, is indicated by (*) . The predicted cleavage site of the leader region (von Heijne, 1986) is indicated with (I). Domains of the tertiary structure (h - helix; e - β sheet; l - loop) were predicted using the algorithms of Sander and Schneider (1991) and the computer program PredictProtein. The cysteine residues involved in the potential disulfide bridges are boxed.

^h
 MKKLLIPLIAAGSLLYPASHA ↓ EDPC[•]KVIM
 ••••••••••
^l ^h
 CMAGKLTGDSGGSE^hCNSAEAAFFNIVKK^hKNKH
 ••••••••••
^l ^h ^l
 GFLPNHTKDARKAFLNE^hCPDNGEGGS^hNQSMI
^h
 SQIISKY^hGKVRL

Table 3.1. Biophysical properties of the Orf104 protein.

	pre-protein	mature protein
Size (aa residues)	104	82
MW (Da)	11,322 ^a	8988 ^b
isoelectric point (pI) ^a	8.95	8.26
mean hydrophobicity ^a	0.83	-0.03
net charge ^a	+5	+3

^a predicted by MacDNASIS computer program^b determined on the mass spectrometer

hydrophobic leader peptide which is cleaved off upon transfer across the membrane (von Heijne, 1986; Izard and Kendall, 1994). It was therefore speculated that the Orf104 protein also contains such a leader peptide and therefore the mature protein identified by gel electrophoresis is smaller than the pre-protein predicted from the DNA sequence of *orf104* (Figure 3.7). A computer search of protein data banks (Swissprot, PIR, EMBL, GenBank) revealed striking similarities between the N-terminal part of the Orf104 protein and the N-terminal parts of some bacterial and non-bacterial exported proteins (Figure 3.8). No significant homology was found, however, for the core of the protein. This similarity to N-terminal leader peptides strongly suggests that the first 20 to 24 amino acids of the Orf104 protein are the signal leader sequence which is being cleaved off upon transfer into the periplasm. This hypothesis is also supported by the hydrophobic nature of the N-terminus of the predicted Orf104 protein (Figure 3.9). The computer program Sigseq (von Heijne, 1986) was used to predict a putative signal sequence for the Orf104 protein and to predict a putative cleavage site for the leader peptide. As indicated in Figure 3.7, the theoretical cleavage site is between the amino acid residues 22 (alanine) and 23 (glutamine). This cleavage site separates the hydrophobic leader peptide from the core protein (Figure 3.9). Protein microsequencing was used to confirm the hypothesis that the first 22 amino acids of the Orf104 protein are indeed the leader peptide. A periplasmic protein fraction was purified by 15% SDS-PAGE, electroblotted onto the Problot PVDF membrane (Applied Biosystems), and the N-terminal part of the Orf104 protein was sequenced on a protein sequencing system (Applied Biosystems). The sequence which was obtained from this analysis completely matched the protein sequence predicted from the DNA template of *orf104*, starting at amino

Figure 3.8. Comparison of the putative leader sequence of the Orf104 protein with the leader sequences of other exported proteins. The protein data banks Swissprot, PIR, GenBank and EMBL were searched using E-mail searches with programs FASTA, BLASTP and BLITZ as described in the Materials and Methods section. Numbers in front of and after the amino acid sequences represent amino acid numbering. (+) represents a positive replacement, (-) represents an identical amino acid residue.

(EC - *Escherichia coli*, PS - *Pseudomonas stutzeri*, BS - *Bacillus subtilis*, HS - *Homo sapiens*; c₅₅₁ - cytochrome c₅₅₁; b₅₆₂ - cytochrome b₅₆₂; nit.red. - nitrate reductase; POTD - periplasmic spermidine/putrescine binding protein precursor; NADH-ox.red. - NADH-ubiquinone oxidoreductase chain 4; PRSA - protein export 33 kDa lipoprotein precursor)

Orf104	1	MKKLLIPLIAAGSLLYLPASHAEDPCKVIN	30
c ₃₅₁ (PS)	1	---+---++-L-GA-A+QP+L-+-	24
b ₃₆₂ (EC)	1	-+-S-+A++-VS---++SS--F-A-	24
nit.red.(PS)	5	-P-+GT-+A+L+--G-AT+---+-DM-	30
PRSA (BS)	1	---+A-AA-T-T-+-A-S-CS+G-	24
1-4 glucanase (BS)	12	---T-+TM-G++AS---	28
POTD (EC)	1	---WSRH-+---+-A-G++-+--+	25
NADH-ox.red.(HS)	1	-L---++-T-MLLP-T+-SKK-	21

Figure 3.9. The hydrophobic profile of the Orf104 protein. The hydropathy for the predicted Orf104 protein was calculated by the method of Kyte and Doolittle (1982) using a window of 13 amino acids. Hydrophobic domains are represented by a hydropathy > 50 , hydrophilic domains are represented by a hydropathy < 50 . The signal peptide cleavage site is indicated with (1).

acid position 23 (Figure 3.7). This confirmed the hypothesis that the first 22 amino acids are indeed a leader region which is cleaved off upon transfer into the periplasm. Also, microsequencing of the N-terminus confirmed the identity of the visualized protein band as the mature Orf104 protein.

The periplasmic fraction of the induced cells was also further precipitated with 80% (w/v) ammonium sulphate to salt out contaminating proteins and most of the lysozyme. It was determined empirically that when using 80% (w/v) ammonium sulphate, the Orf104 protein remains in solution while the contaminating proteins are precipitated. Upon excessive dialysis against 5% acetic acid, the resulting protein sample (0.1 mg/mL) was analyzed on the VG Quattro Triplequadropole Mass Spectrometer (Fisons Instruments). The resulting spectrum is shown in Figure 3.10.

There are three major polypeptide samples (indicated as A, B, and C in upper left corner in Figure 3.10) with molecular masses of $8,988.28 \pm 3.35$, $9,048.54 \pm 4.19$ and $9,106.22 \pm 5.74$ Daltons respectively. The 9,048.54 and 9,016.22 Da species are most likely contaminants, and are less abundant than the 8,988.28 Da species, which represents the Orf104 protein. This result shows that Orf104 is a monomer in solution (at least under the conditions used in this experiment). There is a difference of +134.07 Da between the calculated mass of Orf104 based on the amino acid sequence (8,854.21 Da) and that obtained from the mass spectrometer analysis (8,988.28 Da). This difference is probably even larger (+4.02 Da) since the presence of a disulfide bond accounts for a -2.01 Da reduction in the size of the protein and there are probably two disulfide bonds (four cysteines) involved in the tertiary structure of the Orf104 protein (see below). However, during mass spectrometer

analysis, the disulfide bonds can dissociate. Thus, a difference of 134.07 - 138.09 Da can be attributed to post-translational modification of the Orf104 protein. Several possibilities can be listed for such a modification, based on the masses of the modification groups (Table 3.2). As seen from this table, there are several theoretical modifications which would account for the observed increase in mass. However, some of these modifications are not known to occur in prokaryotes (e.g. sulphation; Creighton, 1993), while others would have to occur several times in order to provide an observed difference in the mass (formylation, acetylation), and they normally occur only once on a given protein (Creighton, 1993). There are three modifications, however, which could take place. The C-terminal addition of histidine would account for the observed increase in mass, and some bacterial proteins are known to be modified by a post-translational addition of an amino acid at the C-terminus (however, the added amino acid residue is usually leucine, phenylalanine, arginine or lysine) (Creighton, 1993). A second possibility is a γ -carboxylation of Glu residues, yielding the γ -carboxyglutamic acid (Gla). This modification is vitamin K-dependent, and modifies Glu residues within the first 40 amino acids of a polypeptide chain (Creighton, 1993). The function of Gla residues is almost invariably linked to the binding of Ca^{2+} ions and an addition of a carboxyl group is necessary for the proper function of the protein. There are three Glu residues within the first 40 amino acids of the Orf104 protein and the molecular mass of the modified protein (8,986.21 Da) would be almost identical to the observed molecular mass ($8,988.28 \pm 3.35$ Da, plus an additional 2.01 or 4.02 Da for the disulfide bond(?)). The third modification could be ADP-ribosylation. Ribosylated proteins are known to be involved in the regulation of cellular processes, and several bacterial endotoxins are ADP-ribosylated

Figure 3.10. Analysis of the mature Orf104 protein on the mass spectrometer. A partially purified protein sample (0.1 mg/ml) was analyzed on a VG Quattro Triplequadropole Mass Spectrometer (flow rate 4 μ L/min) as described in the Materials and Methods section. Three species of polypeptides are indicated in the upper right corner. Sample A ($8,988.28 \pm 3.35$ Da) is most abundant and significant and represents the Orf104 protein.

15-Aug-1994

750 1 (3.016) Cn (Top, 4, Hz); Sm (Min, 2x1.25); Sb (4, 45.00); Sm (Min, 2x1.25); Sm (Min, 2x1.25)

Scan 85+
2.45e7
8988.28±3.36
9048.54±4.19
9108.22±5.74

A:
B:
C:

Table 3.2. The putative post-translational modification of the Orf104 protein.

modification	number of occurrences ^a	molecular mass (Da)	mass of the resulting protein (Da)	limitations ^b
pentose (Rib)	1	132.11	8986.32	-
acetylation	3	42.03	8980.30	N-terminus only
formylation	5	28.01	8994.26	N-terminal Met only
carboxylation of Glu	3	44.00	8986.21	-
histidine at C-terminus	1	137.14	8991.35	-
sulphation+formylation	1+2	80.06, 28.01	8990.29	sulphation only in eukaryotes
myristylation+acetylation				
+removal of last residue	1	210.35, 42.03, -113.15	8993.44	myristyl only at N-terminus
phosphorylation+formylation	1+2	79.98, 28.01	8990.21	formyl at N-terminus only

^a a theoretical number of how many times a particular modification would have to occur in order to account for a predicted MW of a modified protein

^b limitations as to under what circumstances a particular modification can occur. (-) represents no limitation and therefore the highest likelihood that the modification could take place

(Creighton, 1993). However, the half-life of a ribosyl group is only about 1 minute and it is therefore very unlikely that this modification would survive treatment before it was analyzed on a mass spectrometer (Seifter and England, 1990).

When the Orf104 protein was run on a SDS-PAGE-Urea gel (Anderson *et al.*, 1983) in the absence of the reducing agent β -mercaptoethanol, it appears much smaller (~ 7.6 kDa) than expected. The addition of mercaptoethanol to the sample resulted a in shift in the size of the Orf104 band (Figure 3.11). This is due to the presence of four cysteine residues (see also Figure 3.7) which may form disulfide bonds, and in the absence of the reducing agent (mercaptoethanol or DTT), the Orf104 protein will keep its compact globular form, and will thus migrate faster (further) on the polyacrylamide gel than its dissociated form. This may also account for a small (2.01 Da *per* disulfide bond) reduction in the size of the mature protein when analyzed on a mass spectrometer.

The presented results clearly show that the Orf104 protein is a soluble, globular, periplasmic protein of MW $8,988 \pm 3.35$ Da which very likely exists as a monomer. There is no homology between the Orf104 protein and any of the proteins accessible in data banks. There are one or two disulfide bridges involved in the tertiary structure of Orf104, and this protein is post-translationally modified. The first modification involves the cleavage of the N-terminal 22 amino acid leader region. This region is responsible for the proper translocation of the mature Orf104 into the periplasm, and has a strong homology to other bacterial leader peptides. The second modification involves the addition of a modification group(s) onto the mature Orf104 protein. It is not clear, however, to what extent this second modification would affect the proper functioning of Orf104 in the KikA' and/or PRD1^h phenotypes (see

Figure 3.11. SDS-PAGE-Urea analysis of the Orf104 protein in the presence and in the absence of mercaptoethanol. *Klebsiella* cells carrying the plasmid pMH104 were grown and induced as described in the Materials and Methods section and the periplasmic proteins were isolated by the method of Wood (1978). 15% SDS-PAGE-Urea gel was prepared as described by Anderson *et al.* (1983). A MW standard is indicated on the right.

- 1. non-induced cells, no mercaptoethanol**
- 2. IPTG induced cells, no mercaptoethanol**
- 3. IPTG induced cells, + 5% mercaptoethanol**

Part 4, p. for the PRD1^R phenotype). It is also not clear which, if any, of the three above-mentioned post-translational modifications takes place. γ -carboxylation of Glu residues seems to be the most plausible modification based on the likelihood of this modification taking place. This modification may help to explain the role of the Orf104 protein in both the KikA⁺ and PRD1^R phenotypes. γ -carboxylated Glu residues have been shown to be involved in the binding of Ca²⁺ ions (Creighton, 1993). Ca²⁺ ions are known to make bacterial membranes permeable to DNA (Dreiseikermann, 1994), and although the precise mechanism is not known, the Ca²⁺ ions are commonly used in treating bacterial cells for DNA transformation procedures (Cohen *et al.*, 1972). Thus, the Orf104 protein may be a calcium binding protein which is involved in the structure or a function of a membrane channel (see Part 2 and Part 4.)

3.3. Detection and characterization of the Orf70 protein.

The plasmid construct pMH70 (Figure 3.4) was used to overexpress the Orf70 protein in the *Klebsiella oxytoca* host for the purpose of SDS-PAGE analysis. However, even after overnight induction with 1 mM IPTG no unique protein band, representing the Orf70 protein, was detected. There are several explanations for the lack of this protein band on the SDS-PAGE gel:

- (i) if the final product of *orf70* in wild type *kikA* is in fact RNA, there will be no translated polypeptides from the plasmid pMH70 even though it carries a ribosome binding site located at the perfect distance (8 bp) from the putative ATG start codon of *orf70*,
- (ii) the mRNA transcript of *orf70* could be unstable and therefore the amount of translated protein could be insufficient for detection with Coomassie Blue staining, and

(iii) the chemical composition of the putative Orf70 protein could be interfering with the staining procedure. It is known that Coomassie brilliant blue G250 dye interacts primarily with arginine, and to a lesser extent with histidine, lysine, phenylalanine, tyrosine and tryptophan (Compton and Jones, 1985). There are 7 arginines (10%) in the predicted amino acid sequence of Orf70 (Figure 3.12) and it is therefore very unlikely that the absence of certain amino acids would be the reason for not detecting the Orf70 protein on SDS-PAGE gels.

A different experimental approach was therefore used to overcome this obstacle. The plasmid pMH70-T7 carries *orf70* under the control of the strong T7 promoter. This plasmid was transformed into the *E. coli* BL21 (DE3) host which carries an IPTG inducible T7 RNA polymerase gene inserted into its chromosome. It has been documented in the literature (Studier *et al.*, 1990) that after induction with IPTG, the recombinant gene product can contribute more than 50% of the total cellular protein. Also, because the T7 RNA polymerase is resistant to rifampicin, it is possible to selectively pulse-label the protein of interest with ³⁵S-methionine.

Figure 3.13 shows the identification of the pulse-labelled Orf70 protein. There is a protein band unique to the cells carrying the pMH70-T7 plasmid. Since only the polypeptides transcribed by the T7 RNA polymerase can be pulse labeled in this experimental approach, and since there is no detectable band in the control cells, it is very likely that the identified protein band indeed represents the Orf70 protein. It can therefore be concluded that the

Figure 3.12. The predicted amino acid sequence and the tertiary structure of the Orf70 protein. Domains of the tertiary structure (h - helix) were predicted using the algorithm of Sander and Schneider (1991) and the computer program PredictProtein. Potential sites of cAMP/cGMP-dependent kinase phosphorylation (c(P)) and protein kinase C phosphorylation (P) are indicated.

^h
 MLKIPAKLLCAWRASSPAIAAEASVTVLKLLSS
^P ^{c(P)} ^{PP}
 I[S]LKRT[S]TAFYP[T]RRMLGRLFLMNARIMAK
^P
 VEV[T]SR

Figure 3.13. SDS-PAGE (15%) analysis of the ^{35}S -methionine labelled protein samples isolated from *E. coli* BL21(DE3) cells carrying the plasmid pMH70-T7 or pET23d (control). Cells were grown to O.D.(550 nm) ~ 0.5 and were induced with 0.5 mM IPTG for 30 minutes. Pulse labelling with ^{35}S -methionine was done as outlined in the Materials and Methods section. The arrow indicates the Orf70 protein. A MW standard is indicated on the right.

1. pET23d
2. pET23d
3. pMH70-T7
4. pMH70-T7

product of *orf70* is a soluble protein. The inability of this protein to be stained by Coomassie blue still needs to be explained.

Interestingly, the Orf70 protein detected by pulse-labelling has an apparent molecular weight of ~ 9.0 kDa, which is more than the predicted molecular weight of 7,627 Da. It has been shown for several very basic proteins (e.g. histones) that these proteins migrate anomalously during electrophoresis (Hames, 1981; Garfin, 1990). Based on the predicted amino acid sequence of the Orf70 protein (Figure 3.12) this protein is rather basic (pI=12.14, net charge +9), and this could account for the observed anomalous behaviour on SDS-PAGE gels.

Table 3.3 describes some of the biophysical properties of the Orf70 protein as calculated by the MacDNASIS computer program. It is a soluble protein with a mean hydrophobicity of 1.01, an isoelectric point (pI) of 12.14 and a net charge of +9. There is no significant similarity to any proteins accessible through the computer search programs in the protein data banks (Swissprot, EMBL, PIR and GenBank). The Orf70 protein is very likely a cytoplasmic protein, since it is detected in the cytoplasmic fraction (not shown). A computer search in the Prosite database was used to predict putative post-translational modifications of Orf70 and to predict its putative function. As indicated in Figure 3.12, there are four potential protein kinase C phosphorylation sites and one potential cAMP/cGMP-dependent kinase phosphorylation site. It has to be noted, however, that these sites are only potentially biologically significant, since they were predicted based on the signature similarities between the Orf70 protein and known proteins which are modified at the same or similar sites. Direct experimental evidence would be needed to confirm that such predicted modifications can

Table 3.3. Biophysical properties of the Orf70 protein.

Size (aa residues)	70	
MW (Da)	7626 ^a	~9000 ^b
isoelectric point (pI) ^a	12.14	
mean hydrophobicity ^a	1.01	
net charge ^a	+9	

^a predicted by MacDNASIS computer program

^b determined on the SDS-PAGE

really take place.

From the results presented above it can be concluded that the product of *orf70* is a soluble protein located in the cytoplasm. It is possible that this protein can be phosphorylated at several sites and it is very tempting to speculate that the Orf70 protein could function as a signal protein which is required for the proper functioning of the Orf104 protein in the KikA⁺ phenotype.

3.4. Expression of *kikA* - analysis of *kikA* putative promoter sequences.

A paradoxical situation has been observed with *kikA* clones. When the 500 bp *kikA* region, including its putative promoter sequences (Figure 3.1), is cloned into the vector pACYC184 (plasmid pPH0/1; Hengen, 1991), the plasmid can be efficiently transformed into *Klebsiella* and be stably maintained (Figure 3.14). However, when the same region is cloned under the control of the *tac* promoter (plasmid pPH4; Hengen, 1991) and transformed into *Klebsiella*, induction by IPTG results in one of two outcomes: failure of the cells to grow (Hengen, 1991), or loss of the plasmid (Figure 3.14). There has been some concern raised that this phenotype, called KikA⁺ in this thesis, is only a consequence of the overexpression of *kikA*, and is not the same as the killing by the parental plasmid pCU1. As noted above, the expression of both *orf104* and *orf70* of *kikA* is required for the KikA⁺ phenotype, and the relevant genetic regions can be either *in cis* or *in trans*. It seemed unlikely that this would have happened by chance, resulting from the overexpression of both proteins. It was plausible that there is some regulation of the level of expression of *kikA* by another region(s) on the parental plasmid that was deleted upon excising the *kikA* region from the parental plasmid

Figure 3.14. Stability of the plasmids pPH4 (*tac-kikA*) and pPH0/1 (*kikA*) in the presence and in the absence of IPTG in the *Klebsiella oxytoca* UN2979. An overnight cultures were diluted 1:100 and were grown in the presence of 0.5 mM IPTG (or dH₂O, control) at 37°C. The cultures were diluted 1:1000 every 24 hours at which time the samples were withdrawn and plated on LB plates. After overnight incubation at 37°C 100 colonies of each sample were replica plated on LB plates supplemented with appropriate antibiotics. The plates were scored for bacterial growth after overnight incubation. Data were calculated from three independent experiments; standard deviations are indicated as an error bars.

(•) pPH4

(▽) pPH4 + IPTG

(▼) pPH0/1

pCU1.

When *Klebsiella* cells carrying the *kikA* containing plasmid pPH0/1 (KikA⁻ phenotype) were used as recipients in matings with donors carrying the *kikA*⁻ plasmid pCU403, killing occurred (Figure 3.15). Since both plasmids are phenotypically KikA⁻, the observed killing can be only a result of a complementation of *kikA*, the only region of pCU1 present in pPH0/1 and a region present in pCU403 (partial deletion of *kikA*). However, as was shown with plasmid pPH4, the overexpression of *kikA* is also sufficient to produce the KikA⁻ phenotype. A working hypothesis was therefore proposed in which the *kikA* region on the plasmid pPH0/1 can be "activated" by some augmenting region of the plasmid pCU403. This hypothesis is also supported by the presence of two different putative promoters located upstream of the *kikA* coding sequences (Figures 3.1 and 3.2). The promoter P₂ is similar to σ^{70} promoters while the promoter P₁ is similar to σ^{34} (σ^N) promoters. While the majority of sigma factors in Gram-negative bacteria belong to a family of proteins with homology to σ^{70} , sigma factor σ^{34} is quite unique and distinct both structurally and functionally from the σ^{70} family (Merrick, 1993). It has been demonstrated that σ^{34} -holoenzyme transcribes genes whose products have diverse physiological functions (Kustu *et al.*, 1989). Control of transcription at σ^{34} -dependent promoters appears to be accomplished primarily by modulation of the activity state of activator proteins and their abundance (Inouye *et al.*, 1987; Kustu *et al.*, 1989; Buck and Cannon, 1992; North *et al.*, 1993). Each activator allows the σ^{34} -holoenzyme to initiate transcription in response to a distinct physiological signal, such as (i) low oxygen tension, (ii) energy limitation, (iii) limitation of combined nitrogen, or (iv) availability of certain organic solvents (toluene, xylenes) (Kustu *et al.*, 1989). It has been

Figure 3.15. Complementation of the Kik⁺ phenotype.

Klebsiella oxytoca cells carrying either the plasmid pPH0/1 (*kikA*) (2) or pACYC184 (negative control) (1) were spread separately as a lawn of cells on minimal media supplemented with histidine as described in the Materials and Methods section. *E. coli* donor cells carrying the plasmid pCU403 were spotted onto the lawn. After 24 hours, the plates were scored for the Kik phenotype.

1

2

shown that in some cases (e.g. NTRC activator of the *nif* operon of *Klebsiella*), the activator protein can act as an activator or a repressor of transcription (Minchin *et al.*, 1988). The activity of NTRC is modulated by the product of the *ntrB* gene which phosphorylates NTRC under conditions of nitrogen limitation and dephosphorylates NTRC-phosphate under conditions of nitrogen excess. The phosphorylated form of NTRC was shown to be required for transcription *in vitro* (Ninfa and Magasanik, 1986). It is therefore possible that in the absence of the presumptive augmenting (activator) protein (encoded by pCU403) the 500 bp *kikA* region, with its natural promoters, would not express enough protein(s) to allow the development of the KikA⁺ phenotype. Also, since the *orf104* and *orf70* overlap but are not in frame, it is possible to imagine that there must be a fine regulatory mechanism involved in the expression of these two open reading frames, and that this regulation is controlled by a pCU403-encoded protein(s).

To test this possibility, total cellular RNA isolated from the *Klebsiella* cells carrying plasmids pPH0/1, pCU403, pPH0/1 + pCU403, pMH70 and pMH104 was transferred onto a nylon membrane and hybridized with the radiolabelled 500 bp *kikA* region (Figure 3.16). It was found that there are no detectable *kikA* RNAs in the pCU403 background (*kikA* deletion). The amount of *kikA* RNA present in the pPH0/1 carrying cells is very low when compared to plasmids pMH70 or pMH104 (IPTG induced). However, the presence of the plasmid pCU403 together with pPH0/1 had a profound effect on the expression of *kikA* as indicated by the increase in the amount of *kikA* RNA detected (not tested in *E. coli*). There are two mRNA species visible in the extracts isolated from the pPH0/1 carrying cells. It is apparent that the presence of the plasmid pCU403 had a stronger effect on the expression of

Figure 3.16. Northern blot hybridization of *tikA* to total cellular RNA isolated *Klebsiella* cells carrying the plasmids pPH0/1, pCU403, pPH0/1+pCU403, pMH70, and pMH104 respectively. Cells were grown to O.D._{550 nm} ~ 0.5 and were induced with 1 mM IPTG for 3 hours (pMH70 and pMH104) or the cells were grown to O.D._{550 nm} ~ 0.9 (pPH0/1, pCU403, pPH0/1+pCU403). Isolation of total cellular RNA, Northern blot and hybridization was done as outlined in the Materials and Methods section

1. pPH0/1
2. pPH0/1 + pCU403
3. pCU403
4. pMH70
5. pMH104

1 2 3 4 5

the smaller mRNA species. By comparison to the sizes of RNAs detected in the cell extracts carrying plasmids pMH70 and pMH104 it seems that the smaller mRNA corresponds to *orf70* mRNA whereas the larger mRNA corresponds to *orf104* mRNA. This would indicate that the "augmenting" region of the plasmid pCU403 can increase the level of transcription of *orf70*. It has to be noted, however, that without additional experiments such as site-directed mutagenesis of the natural *kikA* promoters it is not possible to ascribe a particular *orf* to the transcript regulated by a particular promoter.

To directly test the effect of pCU403 on the promoters of *kikA*, the putative promoter regions P_1 , P_2 and the whole region $P_{1,2}$ were cloned into the cloning vector pNEB193 (New England BioLabs). These clones were confirmed to have wild type promoter sequences by nucleotide sequencing and the promoters were then recloned into the Cm^R promoter searching vector pKK232-8 (Brosius, 1984), yielding the plasmids pKK- P_1 , pKK- P_2 and pKK- $P_{1,2}$ respectively (Figures 3.17, 3.18, 3.19). If a DNA fragment containing a promoter is inserted into pKK232-8, cells carrying this plasmid acquire resistance to chloramphenicol (Cm^R).

Promoter region P_1 extends from the nucleotide 63 (primer MH3) to nucleotide 164 (primer MH4). It contains an entire putative promoter P_1 including a putative ribosome binding site. It also contains a start codon of *orf104*. This codon, however, was mutated by introducing two base substitutions at positions 158 (G → C) and at position 161 (G → C) (ATG AAG → ATC AAC). Upstream of the putative promoter P_1 the P_1 region includes a -10 box of the putative promoter P_2 . It lacks, however, a -35 sequence of P_2 . Promoter region P_2 extends from the nucleotide 21 (primer MH1) to nucleotide 115 (primer MH2). The

Figure 3.17. Construction of the plasmid pKK-P₁, carrying the putative promoter P₁ of *kikA*. PCR cloning was used to introduce restriction sites on 5' (*EcoRI*) and 3' (*BamHI*) ends of the fragment. The primers used for PCR are indicated in small, underlined numbers. The *EcoRI*-*BamHI* PCR fragment was inserted into the plasmid pNEB193, yielding the plasmid pNEB-P₁. The plasmid pNEB-P₁ was then linearized with *EcoRI*, filled in, and cut with *BamHI*. The DNA fragment carrying the putative promoter sequence was then inserted into *SmaI* and *BamHI* sites of the plasmid pKK232-8, yielding the plasmid pKK-P₁. *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon.

AAGCTTGCATGCCTGCAGGTCGAGGTTTGTGTGCGAATATATTGCAGGCG
 ATATAGGTTACAAATCCATTATAACGCAGCGTTATTATTAATATTACTT
 ACCTTTATCAACATTCTGCCGAGTGTATCTGAACAACTGTAAAGGAC
 AAAAAATGAAGAACTCTTA
 ORF104

PCR

Figure 3.18. Construction of the plasmid pKK-P₂, carrying the putative promoter P₂ of *kikA*. PCR cloning was used to introduce restriction sites on 5' (*EcoRI*) and 3' (*BamHI*) ends of the fragment. The primers used for PCR are indicated in small, underlined numbers. The *EcoRI*-*BamHI* PCR fragment was inserted into the plasmid pNEB193, yielding the plasmid pNEB-P₂. The plasmid pNEB-P₂ was then linearized with *EcoRI*, filled in, and cut with *BamHI*. The DNA fragment carrying the putative promoter sequence was then inserted into *SmaI* and *BamHI* sites of the plasmid pKK232-8, yielding the plasmid pKK-P₂. *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon.

AAGCTTGCATGCCTGCAGGTCGAGGTTTGTGTGCGAATATATTGCAGGCG
 ATATAGGTTACAAATCCATTATAACGCAGCGTTATTATTATTAATATJACTT
 ACCTTTATCAACATTCTGGCGAGTGTATATCTGAACAAACTGTTAAGGAC
 AAAAAATGAAGAAACTCTTA
 ORF104

PCR

Figure 3.19. Construction of the plasmid pKK-P₁₋₂, carrying the putative promoters P₁ and P₂ of *kikA*. PCR cloning was used to introduce restriction sites on 5' (*EcoRI*) and 3' (*BamHI*) ends of the fragment. The primers used for PCR are indicated in small, underlined numbers. The *EcoRI*-*BamHI* PCR fragment was inserted into the plasmid pNEB193, yielding the plasmid pNEB-P₁₋₂. The plasmid pNEB-P₁₋₂ was then linearized with *EcoRI*, filled in, and cut with *BamHI*. The DNA fragment carrying the putative promoter sequence was then inserted into *SmaI* and *BamHI* sites of the plasmid pKK232-8, yielding the plasmid pKK-P₁₋₂. *rrnB* is a transcriptional terminator from an *E. coli* rRNA operon.

AACCTTGCATGCCTGCAGGTCGAGGTTTGTTCGAATATATTGCAGGCC
 ATATAGGTTACAATCCATTATAACGCAGCGTTATTATTAATATTACTT
 ACCTTTATCAACATTCTGGCGAGTGTATATCTGAACAACTGTAAAGGAC
 AAAAAATGAAGAACTCTTA
 ORF104

PCR

putative -26 box of the P_1 promoter starts at the position 116 and is not part of the promoter region P_2 . Promoter region $P_{1,2}$ extends from the nucleotide 21 (primer MH1) to nucleotide 164 (primer MH4) and it contains both putative promoters P_1 and P_2 , ribosome binding site as well as mutated start codon of *orf104*. The promoter regions P_1 and P_2 were synthesized to be bigger than just the core promoter sequences so that a potential effect of DNA-binding proteins in the vicinity of each promoter could be observed. Promoter constructs were transformed into *Klebsiella* and *E. coli* cells, with and without the plasmid pCU403, and screened for the level of chloramphenicol resistance as outlined in the Materials and Methods section.

As shown in Figure 3.20, the presence of pCU403 had a strong effect on the level of expression from both *kikA* promoters. The strength of the promoter P_1 was reduced by 33% when pCU403 was present in *Klebsiella*. On the other hand, the expression from the P_2 promoter in the same host was increased by 50% in the presence of pCU403. When the whole region $P_{1,2}$ was tested in *Klebsiella*, the presence of pCU403 increased expression by almost 100%. This result is consistent with the finding that the total amount of *kikA* RNA (transcribed from $P_{1,2}$ region in plasmid pPH0/1) has increased in the presence of pCU403 (Figure 3.16). However, the overall promoter strength of this region was much less than P_1 or P_2 alone. This is probably due to the fact that the two promoters might interfere with each other when cloned with the reporter gene.

When the same constructs were tested in *E. coli* strain DH5 α , a different pattern was observed (Figure 3.20). The expression from the P_1 promoter was increased by 50% in the presence of pCU403. The expression from the P_2 promoter was, however, decreased by 95%

Figure 3.20. Analyses of the strength of the putative promoters of *kikA*. *Klebsiella oxytoca* UN2979 (■) and *E. coli* DH5 α (□) cells with or without the plasmid pCU403, and carrying either the promoter plasmid pKK-P₁, pKK-P₂ or pKK-P_{1,2}, were grown to O.D.(550nm) ~ 0.7 and were spotted (5 μ L) onto LB agar plates supplemented with different concentrations of chloramphenicol as described in the Materials and Methods section. The plates were grown overnight at 37°C and scored for bacterial growth. Bars indicate the highest concentration of chloramphenicol at which the tested bacterial strains showed positive growth after 20 hours. Horizontal line represents a background level of resistance to chloramphenicol (< 2 μ g/mL) for both *E. coli* and *Klebsiella*.

in the presence of pCU403. The whole region $P_{1,2}$ was not influenced by the presence of pCU403. It is also interesting to note that while the strength of P_1 in *Klebsiella* and *E. coli* is almost the same, there was a profound difference when the promoter P_2 was tested. The level of expression from P_2 in the *E. coli* host was 20 fold higher than in *Klebsiella*.

Based on these results it can be concluded that:

- (i) both promoters, P_1 and P_2 , are functional promoters in both *Klebsiella* and *E. coli*,
- (ii) promoter P_1 is *down-regulated* by the plasmid pCU403 in *Klebsiella* and *up-regulated* by the same plasmid in the *E. coli* DH5 α host,
- (iii) promoter P_2 is *up-regulated* by the plasmid pCU403 in *Klebsiella* and *down-regulated* by the pCU403 in the *E. coli* DH5 α host,
- (iv) the entire promoter region $P_{1,2}$ is *up-regulated* by the plasmid pCU403 in *Klebsiella* and induces no change in *E. coli* DH5 α ,
- (v) the action of the augmenting region of pCU403 on the *kikA* region is at the level of transcription. It is very likely a DNA-binding protein which can bind somewhere in the vicinity of the P_1 and P_2 promoters and thus modulate their activity.

Even though there is no direct evidence to show which promoter transcribes which open reading frame, based on the results presented it seems plausible that the promoter P_2 is involved with the transcription of *orf70*. Promoter P_2 is up-regulated in *Klebsiella* and it seems that there is more of the *orf70* transcript than of the *orf104* transcript when plasmid pCU403 is present with pPH0/1 (see mRNA analysis above and Figure 3.16). The role of the augmenting region of the plasmid pCU403 would then be to increase the level of expression of *orf70*. This regulation will achieve the proper molecular ratio of products of both open

3 of/de 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

reading frames which is required for the KikA⁺ phenotype. Therefore, the plasmid pPH0/1 alone is not sufficient for producing the KikA⁺ phenotype, and only if the augmenting region is also provided will this result in the KikA⁺ phenotype.

It is not known why the P₁ and P₂ promoters behave differently in *Klebsiella* and in *E. coli* with respect to the augmentation by the plasmid pCU403. It seems odd, that the same promoter will respond to the presence of pCU403 differently in two different hosts. It is possible, however, that there is a difference in *Klebsiella* and *E. coli* species with respect to the production, stability and availability of the "augmenting" protein. Also, since the natural *kikA* promoter region was dissected into promoter regions P₁ and P₂, caution must be taken when making any conclusions from the experiments presented above. It is possible that the entire promoter region contains several binding sites for the "augmenting" protein(s) which allow for the fine regulation of the transcription. By separating the P₁ region from P₂ this organization might have been destroyed. It is also possible that the Orf70 protein could play some role in the regulation of its own expression, and/or the expression of the Orf104 protein. It was shown in this chapter, that the Orf70 protein is located in the cytoplasm and might be phosphorylated. It is tempting to speculate, that this protein has a dual role: (i) regulation of the expression of the *kikA* locus, and (ii) direct involvement in the KikA⁺ phenotype.

5. Location of the augmenting region.

As described and discussed above, the presence of the plasmid pCU403 had an effect on the transcription of the *kikA* RNAs. It has been hypothesized that plasmid pCU403 carries an "augmenting" region which regulates the expression of *kikA*. Attempts were made to

identify and characterize this region.

The test for "augmentation" was performed by complementation analysis. Plasmids containing different fragments of the *tra* region were mobilized into *Klebsiella* cells carrying plasmid pUC1318*kikA* using one of the *oriT* plasmids pCU801 (Rotheim *et al.*, 1988) or pVT149-1 (Rodríguez de González, 1994) as conjugative helper plasmids in a spot mating. Neither of these two helper plasmids are themselves transferred into *Klebsiella* as their respective *oriT* regions have been deleted. The clear zone of inhibition would therefore indicate that the cloned fragment carries an "augmenting" region. The results are summarized in Figure 3.21.

Thatte (1984) showed previously that the plasmid pCU109 (pCU1 coordinates 27.5 to 11.5 cloned into pACYC184) was *Kik*⁺, and he concluded that all the plasmid regions required for *Kik*⁺ are within this region of pCU1. It was therefore concluded here that the "augmenting" region must be also located within this region of pCU1.

The *KpnI* deletion derivative of the plasmid pCU403, pCU403-1 (Rodríguez de González, 1994) was tested for the "augmentation" of *kikA*. This plasmid lacks the part of the *tra* region between coordinates 33.8 and 4.2 (Figure 3.21). When mobilized with the help of the pVT149-1, the plasmid pCU403-1 was able to augment the *kikA* region for the *Kik*⁺ phenotype. This result suggests that the "augmenting" region must be located outside the *KpnI* coordinates 33.8 to 4.2 on the pCU1 map. Plasmid pCU403-1 was used instead of a similar *KpnI* deletion derivative of pCU109 because it had been observed by Rodríguez de González (1994) that when such a *KpnI* deletion derivative of pCU109 was transferred into *Klebsiella*, its DNA was always found to be rearranged.

Plasmid pCU57Δ14 (Paterson and Iyer, 1992) extends from coordinate 3.7 to 9.55. When mobilized with the help of the pCU801, this plasmid was not able to augment the *kikA* region to give a Kik⁺ phenotype, thus eliminating the region from 3.7 to 9.55 as a putative augmenting region.

Plasmid pCU56Kan (Figure 3.21) was also found not to augment the Kik⁺ phenotype. Since this plasmid extends from the pCU1 coordinate 7 to 11.5 (including *kikB*) it must be concluded that this region alone is insufficient for the augmentation of the *kikA* region.

The 2.5 kb region of pCU1 (coordinates 29.8 to 32.3) from the Tn5 insertion in *traA* (pCU171, Thatte *et al.*, 1985b) to 160 bp after the *kikA* region was recloned from the plasmid pAAG8 (Rodríguez de González, 1994) into the plasmid pCU56Kan, yielding the plasmid pCU56Kan-G8 (Figure 3.22). Plasmid pCU56Kan was constructed by inserting a Km^R *Sall* cassette (Pharmacia) into a *Sall* site of the chloramphenicol gene of the plasmid pCU56 (Thatte *et al.*, 1985b). In an independent study, Rodríguez de González (1994) found that the plasmid pAAG8 cannot be transformed into *Klebsiella* and since this plasmid also carries the *kikA* region, the region between coordinates 30.5 to 32.2 seemed to be a good candidate for the "augmenting" region. However, as indicated in the Figure 3.21, after mobilization into *Klebsiella* carrying the *kikA* region no zone of inhibition was observed. It must be therefore concluded that the "augmenting region" is not located in the region between *kikA* and *traA*.

The plasmid constructs used in the complementation analysis to identify the "augmenting" region covered the entire region of pCU1 present in pCU109 extending from pCU1 coordinate 27.5 to coordinate 11.5. The small region between the Tn5 insertion in pCU171 (coordinate 32.3) and the *KpnI* site at coordinate 33.8 was implicated by the results

with pCU403-1 to potentially carry the "augmenting" region. However, no overlapping plasmid construct was tested to verify this possibility. It was shown that in the closely related plasmid pKM101 there are three open reading frames, *traM*, *traA* and *traB* in this region and that they might be transcribed as a polycistronic transcript from one promoter (Pohlman *et al.*, 1994). It was shown by the comparison of the nucleotide sequences that the *tra* regions of plasmids pKM101 and pCU1 are almost identical (Rodríguez de González, 1994). The Tn5 insertion in pCU171 is located within the *traM* open reading frame (Rodríguez de González, 1994) and thus should also prevent transcription of both *traA* and *traB*. It was also shown in an independent study (Rotheim *et al.*, 1988) that the products of the pCU1 *tra* genes are not required for the Kik⁺ phenotype. In this study, plasmids with Tn5 insertions in each of the *tra* complementation group were mobilized into *Klebsiella* and were shown to confer the Kik⁺ phenotype. Taken together these results indicate that it is very unlikely that the pCU1 region between the coordinates 32.3 to 33.8 carries the "augmenting" region.

The results presented and discussed thus show that none of the pCU1 regions when tested alone carry the "augmenting" region. It is possible then that the function of the "augmenting" region requires products of more than one open reading frame and that these are located in different loci of pCU1. Thus, when separated by cloning their combined function was destroyed.

The evidence presented in this thesis suggests that even though the expression of *kikA* in *Klebsiella* from an artificial promoter is sufficient to give Kik⁺ phenotype, the *kikA* region alone with its natural promoters is not harmful to the cell and can be stably maintained. It was also shown that additional region, here called "augmenting" region, is required to regulate

expression of *kikA* at the level of transcription. The evidence also indicates that the "augmenting" region may consist of more than one genetic locus. The regulation of *kikA* expression may then be similar to that of *kil* and *kor* loci of plasmid RK2 where the combined action of several regulatory genes (*kor*) are required for the control of the *kil* genes in *E. coli*. In both pCU1 and pKM101, there is preliminary evidence suggesting that the *kikA* - *traA* region is genetically complex and has several potentially lethal genes (Rodríguez de González, 1994; Moré and Winans, 1994). A full understanding of the *kil* genes of *E. coli* and the *kik* genes of *Klebsiella* (including the augmenting region) is likely to need an unravelling of this complexity.

Figure 3.21. Identification of the "augmenting" region. The *tra* complementation groups are based on those of Thatte *et al.* (1985a,b), the *oriT* region is based on sequence analysis of Paterson and Iyer (1992). The position of *kikA* and *kikB* regions is based on the data from Thatte *et al.* (1985a,b), Hengen (1991) and Rodríguez de González (1994). The region between coordinates 32.0 to 35.0 is enlarged and showed in the upper left corner. The positions of the open reading frames *traM* and *traA* is based on the sequence analysis of Pohlman *et al.* (1994) for plasmid pKM101. The black triangle indicates position of the Tn5 insertion in pCU171. The solid black lines represent plasmid pCU1 regions present in various plasmid derivatives, the dotted lines represent deletions. The plasmid pAAG8 (Rodríguez de González, 1994) which was used to construct pCU56Kan-G8 extends from coordinate 29.8 to 32.3. The ability of these regions to augment the *kikA* region to give Kik⁺ phenotype (Augmentation), or the ability of these regions to confer the Kik⁺ phenotype (Killing) in *Klebsiella* is indicated below the pCU1 map (+ positive; - negative; nt - since plasmids pCU171 and pCU109 confer Kik⁺ phenotype, the augmentation cannot be tested)

Plasmid	Augmentation	Killing
pCU109	nt	+
pCU171	nt	+
pCU403	+	-
pCU403-1	+	-
pCU56Kan	-	-
pCU56Kan-G8	-	-
pCU57Δ14	-	-

Figure 3.22. Construction of the plasmid pCU56Kan-G8. 2.5 kb region of pCU1 (plasmid coordinates 29.8 to 32.3) was recloned from the plasmid pAAG8 (Rodríguez de González, 1994) as a *HindIII* (filled) - *EcoRI* fragment into *EcoRI* and *ScaI* sites of the plasmid pCU56Kan. Plasmid pCU56Kan itself was created by inserting the Km^R cassette (Pharmacia) into a *Sall* site of pCU56 (Thatte *et al.*, 1985b).

Part 4.

Relationship between the different phenotypes conferred by the *kikA* region.

Three Tn5 insertion derivatives of pCU1 (66, 167, 184) were found to have an insertion in the *kikA* region that Thatte *et al.* (1985b) placed outside the *tra* region. An interesting phenotype of all of these derivatives is that in addition to the Kik⁻ phenotype they were also resistant to the bacteriophage PRD1, a phage known to attach to the N pilus tip (Olsen *et al.*, 1974) and initiating infection following such attachment. These Kik⁻ mutants have been shown to not affect conjugative ability of pCU1 in *E. coli* - *E. coli* matings. Thatte *et al.* (1985) also showed that these three mutants or deletions of *kikA* derived from them were sensitive to the filamentous pilus-specific phage IKe and they produced N type pili. This behaviour is in contrast to all other Tra⁻ mutants of pCU1 that did not produce N pili and were therefore PRD1^R.

To confirm that the inactivation of *kikA* does not interfere with mating and the recovery of transconjugants in *E. coli*, bacterial matings between *E. coli* donors and recipients were performed (Table 4.1) As shown in this table, the deletion of *kikA* in the plasmid pCU403 (deletion derivative of pCU66, see Figure 1, p.6) did not affect conjugation as compared to wild type plasmid pCU1 thus confirming the observation of Thatte *et al.* (1985b). A different result was obtained, however, when *Klebsiella* was used as a recipient. As shown in Table 4.2 there is about a hundredfold reduction in the frequency of transfer between the plasmids pCU1 and pCU403. Moreover, when the *kikA*-carrying plasmid pPH10/1 (*oriT*⁺) was used *in trans* with plasmid pCU403, the frequency of transfer of pCU403 was restored to that of pCU1. The same result was obtained if plasmid pMH104 carrying only *orf104* of *kikA* was used with pCU403. However, the presence of *orf70* alone had no effect on the conjugal ability of pCU403. These results add a third phenotype to genes in this region

Table 4.1. Frequencies of conjugation in *E. coli* - *E. coli* matings carrying the plasmids pCU1 (wild type) and pCU403 (*kikA*⁻).

donor plasmid	frequency ^a
pCU1	$2.11 \times 10^{-1} \pm 0.016$
pCU403	$1.86 \times 10^{-1} \pm 0.034$

^a frequency of conjugation (transconjugants per donor) was measured in three independent matings

Table 4.2. The effect of *kikA* on the conjugation in *E. coli* - *Klebsiella* matings

donor plasmid(s)	<i>kikA</i> orfs	frequency of transfer *
none	-	-
pCU1	<i>orf104, orf70</i>	$2.38 \times 10^{-2} \pm 1.09 \times 10^{-2}$
pCU403	-	$1.50 \times 10^{-4} \pm 1.76 \times 10^{-5}$
pCU403 + pPH0/1	<i>orf104, orf70</i>	$4.24 \times 10^{-2} \pm 2.49 \times 10^{-2}$
pCU403 + pMH104	<i>orf104</i>	$2.38 \times 10^{-2} \pm 1.84 \times 10^{-3}$
pCU403 + pMH70	<i>orf70</i>	$4.64 \times 10^{-4} \pm 2.81 \times 10^{-4}$
pCU403	pPH0/1 in <i>Klebsiella</i>	$3.70 \times 10^{-5} \pm 1.85 \times 10^{-5}$

* frequency of conjugation (transconjugants per donor) was measured in three independent matings

and indicate that the *kikA* region, more specifically *orf104* of *kikA*, plays a positive role in bacterial matings with *Klebsiella*, but not with *E. coli* as recipients. This finding seems paradoxical. On the one hand, the presence of *kikA* reduces the viability of *Klebsiella* and on the other hand its presence seems to stimulate conjugal transfer to the same species. There are several levels where *orf104* could be required for the recovery of pCU1-carrying *Klebsiella* transconjugants - mating pair formation, conjugal DNA transfer, or establishment and maintenance of the plasmid in the new host. To shed light on this problem, *Klebsiella* carrying the *kikA* plasmid pPH0/1 was used as a recipient in matings with pCU403. As indicated in Table 4.2, the presence of *kikA* in the recipient did not increase the frequency of transconjugants carrying pCU403. On the contrary, this frequency was even lower than without pPH0/1. This decrease in frequency of transfer, however, is due to the complementation of pCU403 and pPH0/1 to give the Kik⁺ phenotype (discussed in details in Part 3 of Results and Discussion). This experimental result suggests that to have stimulatory effect on the transfer of pCU403, the Orf104 protein must be present in the donor, not the recipient, thus eliminating the possibility that *kikA* is involved in the establishment or maintenance of pCU403 in *Klebsiella*. The precise role of *orf104* of the *kikA* region in the conjugal transfer is not known. Based on the evidence that *kikA* must be present in donor and that it is the periplasmic Orf104 protein which is important for plasmid transfer it can be hypothesized that it plays a role during plasmid DNA transfer *via* mating bridge and that this process is somehow host-specific. This role of the Orf104 protein in DNA transfer is also supported by the findings that it can facilitate bacteriophage PRD1 genome entry presented below. It is possible that *kikA* may be part of an extended *tra* region of pCU1, the extension

being required only in mating with some bacterial species (e.g. *K. oxytoca*) but not in others (e.g. *E. coli* K-12). This observation is both novel and interesting as most recent detailed studies on bacterial conjugation in which the frequencies of transfer are assessed have tended to use donors and recipients of the same species. The role of the recipient in bacterial conjugation is not understood. Although *orf104* is shown here to stimulate bacterial conjugation in a recipient-specific manner, it has been shown already (Parts 2 and 3) to be part of the *kikA* system.

It may be noted that the KikA⁻ mutation represents a unique class of mutation which affects infection by the pilus-specific phage PRD1 but does not affect conjugation in *E. coli*. Several of the PRD1-resistant mutants of the P tra system of conjugative plasmid RP1 (RP4) have been described (Kotilainen *et al.*, 1993). All of the 54 mutations leading to PRD1-resistant phenotype resided in the transfer region of the plasmid, as was the case for most of the mutants of pCU1.

When the *kikA* carrying plasmid pPH4 (*kikA* under the control of the inducible *tac* promoter) was transformed into a host already harbouring the plasmid pCU403, the PRD1-sensitive phenotype of pCU403 was restored (Table 4.3). This was observed when either *E. coli* or *Klebsiella* hosts (not shown) were used. Restoration of PRD1 sensitivity was achieved even without the exogenous induction of *kikA* indicating that only a small amount of KikA protein(s) is required for susceptibility to PRD1. The same result was obtained when another *kikA* derivative, pPH0/1 (Hengen, 1991) was used *in trans* with pCU403 (Table 4.3). The plasmid pPH0/1 carries the entire *kikA* region including its natural promoters; however, it

Table 4.3. Relevant phenotypes of plasmids with respect to PRD1.

plasmid(s)	<i>kikA</i> orfs	plaquing ^a
none	-	-
pCU1	<i>orf104, orf70</i>	+
pCU403	-	-
pCU403 + pPH4	<i>orf104, orf70</i> ^b	+
pCU403 + pPH0/1	<i>orf104, orf70</i> ^c	+
pCU403 + pMH104	<i>orf104</i> ^b	+
pCU403 + pMH70	<i>orf70</i> ^b	-

^a as determined by the ability of the phage to form plaques on the lawn of bacteria tested (outlined in the Materials and Methods section - Phage infection test).

^b relevant open reading frames are under the control of an inducible *tac* promoter

^c relevant open reading frames are under the control of their natural promoters

lacks the inducible *tac* promoter.

From the genetic analysis described and discussed in the second Part of Results and Discussion, it was concluded that there are two open reading frames, *orf104* and *orf70*, in the *kikA* region. These two open reading frames were separated by PCR-directed mutagenesis (see Part 3, p.102 to p.108) and used to complement pCU403 to determine which of the two open reading frames is involved in the PRD1^s phenotype. The clones pPH4, pMH104, and pMH70 were transformed into an *E. coli* strain C600 carrying the plasmid pCU403. As Table 4.3 indicates, the clones pPH4 and pMH104 were able to restore the PRD1^s phenotype. The clone pMH70, however, was unable to do so. Since the plasmid pMH104 carries only the *orf104* sequence, it is very likely that only the product of this *orf* is involved in PRD1 infection. Interestingly, while the role of *orf104* in stimulating conjugation is *Klebsiella*-specific, its role in conferring PRD1-sensitivity seems to lack such specificity. As discussed in an earlier section (Part 3, p.108 to p.121) the polypeptide Orf104 was shown to be located in the periplasmic space. The size and predicted biophysical properties of this polypeptide (soluble polypeptide located in the periplasm) indicate that it is not likely to be the receptor protein for the PRD1 phage.

IncN plasmids also confer sensitivity to the filamentous phage IKe (Khatoon *et al.*, 1972) and it was suggested by Bradley (1979) that PRD1 and IKe share the same receptor for their adsorption (presumably the conjugative pilus tip, encoded by the Inc N, P or W group plasmids). However, it has been shown previously, that only the infecting ability of PRD1, and not that of IKe, is affected by the mutation in *kikA* (Thatte *et al.*, 1985b). Therefore, it seems plausible that IKe and PRD1 have two different modes of introducing

their respective genomes into the host.

Bradley and Whelan (1989) have demonstrated that the chromosomal mutation *tolQ* prevented the infection of tip-adsorbing filamentous phages, including IKe. The same mutation, however, did not affect infection by shaft (side)-adsorbing filamentous phages or some other phages such as PRD1. Similar to the mutation in *kikA*, the chromosomal *tolQ* mutation did not affect the conjugative ability of *E. coli* carrying the plasmids tested. It has been proposed (Bradley and Whelan, 1989) that after the adsorption of the phage to the conjugative pilus tip, the pilus retracts and the phage virion is pulled through a molecular assembly in the outer membrane. Once in the periplasm, the capsid is removed and the virus DNA is further translocated through the inner membrane by the *tolQRAB* gene products. Phages with a large diameter (such as PRD1), or phages which attach at the side of the pilus, cannot be pulled through the outer membrane and they must therefore have a different mode of infection.

The *tolQ* mutation was originally described as a mutation which rendered the cell tolerant to group A colicins (Nagel De Zwaig and Luria, 1967). It was found later that the products of the *tol* operon are also required for the translocation of the phage DNA across the bacterial membrane (Sun and Webster, 1986). The mutations in the *tol* operon have a pleiotropic phenotype (Dreiseikermann, 1994). They are hypersensitive to certain antibiotics and detergents, tolerant to group A colicins and inhibit infection by the tip-adsorbing phages. The precise function of the Tol proteins is not known, but it seems that they stabilize the outer membrane and are perhaps involved in membrane assembly (Levengood-Freyermuth *et al.*, 1993).

There is a second, analogous transport system in the bacterial cell, called the TonB transport system (Postle, 1990). This system is a high-affinity active transport system for vitamin B₁₂, iron-siderophore complexes, and the group B colicins. Mutations in *tonB* are also tolerant to bacteriophages ϕ 80 and T1. Although the Tol and Ton transport systems are independent of each other and have affinities for different substrates, it was shown that they can cross-complement each other (Braun, 1989; Braun and Hermann, 1993), suggesting their common evolutionary origin.

Since *tolQ* is not necessary for PRD1 infection, I tested whether *tonB* is required for PRD1 infection. The *tonB* mutant strain was transformed with the plasmid pCU1 and the resulting strain used in the PRD1 infection test. As shown in Figure 4.1 the mutation in *tonB* had no effect on PRD1 infection. It can be therefore concluded that the bacteriophage PRD1 requires neither Tol nor Ton transport systems for infection of the host cell.

It was shown by electroporation and transfection experiments that the role of the plasmid in the PRD1 phage infection is restricted to the entry of the phage (Lyra *et al.*, 1991). When the purified genome of PRD1 was electroporated into a host without any plasmid, lysis and phage propagation occurred (Lyra *et al.*, 1991). It seems then, that the mutation in *orf104* must interfere either with the binding of the phage to the surface of the cell or with the injection of the phage genome into the host.

Figure 4.1. Bacteriophage PRD1 infection of isogenic *E. coli* strains AB2847 and BR158 (*tonB*) carrying the plasmid pCU1.

- 1. AB2847/pCU1**
- 2. BR158(*tonB*)/pCU1**

1

2

To elucidate which part of the PRD1 life-cycle is dependent on the presence of *orf104*, the phage DNA was labelled with $^{32}\text{P}\text{-H}_3\text{PO}_4$ *in vivo*. Radiolabelled phage was used in the binding experiments with cells carrying either the plasmid pCU1 or the plasmid pCU403 (Figure 4.2). Phage was found bound to the cells carrying either plasmid, indicating that the mutation in the *kikA* gene does not affect phage binding to the receptor. In a separate experiment, a longer incubation time was used to allow the entry of the phage genome into the host. Upon treatment with lysozyme, the radioactivity was found in the spheroplasts of the pCU1 carrying cells, but not the pCU403 carrying cells (Figure 4.2). As seen in Figure 4.2, the amount of radioactivity found in the periplasmic fraction of pCU1 carrying cells was same as that of the negative control cells (no plasmid) while the amount of radioactivity found in the periplasmic fraction of pCU403 carrying cells was about 4-fold higher. When the radioactivity associated with the spheroplasts was analyzed it was found that the amount found in pCU1 carrying cells was about 5.5-fold higher than that found in the control cells. The radioactivity found in the pCU403 carrying spheroplasts was 2-fold less than that of pCU1 carrying cells, but it was about 2.5-fold higher than that of the control cells. This could be attributed to the fact that during the lysozyme-EDTA treatment some of the radiolabeled PRD1 DNA remained trapped with the spheroplasts. This, however, did not happen in the case of pCU1 carrying cells since the lysozyme-EDTA treatment took place after the PRD1 DNA was already transported inside the spheroplasts. Comparison of the pCU403-carrying spheroplasts-associated and periplasm-associated radioactivity clearly shows that the majority of the PRD1 DNA was released by the lysozyme-EDTA treatment. These results indicate that

Figure 4.2. Bacteriophage PRD1 infection (binding and DNA injection) of *E. coli* C600 carrying plasmids pCU1, pCU403 and without any plasmid. The phage binding and phage DNA injection assays were performed as outlined in the Materials and Methods section. Binding (□) was measured as radioactivity associated with total cells. The DNA injection (■) was measured as radioactivity associated with spheroplasts whereas (▣) represents radioactivity associated with the cell surface and periplasmic space. Histograms represent average data from three independent experiments; standard deviations are indicated as an error bars above each bar.

PRD1 is capable of binding to the host cell carrying pCU403 but is unable to inject its genome into the cytoplasm. Thus, the gene product of *orf104* must play an important role in the post-binding entry of the viral genome into the cell. I propose to call this gene *ppi* (PRD1 phage infection).

Since the mutation in *orf104* does not affect the binding of the phage to the receptor, there are two possible hypotheses as to the function of Orf104 protein.

According to the first hypothesis, the Orf104 protein may be required to provide a signal to the bound phage which will trigger the release of the DNA. Olsen *et al.* (1977) purified the somatic receptor for PRD1 and have shown that the binding of the purified receptor to the radiolabelled phage did not trigger the release of the phage DNA. The existence of another protein(s) involved in the receptor complex which would initiate the injection of DNA was therefore suggested. The Orf104 protein, however, is found in the periplasm and not in the outer membrane as would be expected for a receptor complex protein. It is possible that after the tight binding to the receptor, a part of the phage-receptor complex protrudes inside the periplasm where the signal for DNA release could then be mediated by the periplasmic Orf104.

The second hypothesis assumes that the phage PRD1 binds to the cell surface and will release its genome into the periplasm. The phage PRD1 genome, however, consists of double-stranded DNA with a 5'-linked terminal protein at each terminus which are not dissociated before or during entry (Bamford and Mindich, 1984). Even though PRD1 uses the same surface receptor as the phage IKe, it is possible that its genome may be too bulky to use the same route as the relatively "slim" genome of the phage IKe. The IKe genome is single-

stranded DNA; the PRD1 genome is double-stranded with a terminally-attached proteins. The role of the Orf104 protein could then be to "stretch" or open the already existing channel in the membrane to allow translocation of the PRD1 genome. This model is also supported by the fact that the infection of PRD1 is resistant to mutations in both *tol* and *ton* operons.

A similar situation may exist for the phage lambda. The phage lambda binds to the LamB receptor which is located in the host outer membrane. LamB is also a component of the maltose transport system (Dreiseikermann, 1994). The three subunits of the LamB protein were shown to form a channel for the selective transport of maltose and maltodextrins (Luckey and Nikaido, 1980). It was therefore speculated that the same pore may also be a channel for lambda DNA translocation across the bacterial membrane. When the LamB was reconstituted into the liposomes, phage lambda injected its DNA into the liposome. The inner diameter of pore formed by LamB (estimated to be 0.7 nm), however, is not sufficient for the translocation of double-stranded DNA (Dreiseikermann, 1994). It was speculated that the interaction of the lambda tail proteins with LamB would lead to an extension of the LamB pore. The hypothesis of Orf104 as a protein involved in the function of a channel is also supported by the fact that when *kikA* is expressed in *Klebsiella*, the membrane potential is dissipated suggesting that a channel in the membrane is opened (see second part of Results and Discussion, p.77 to p.98). It has been shown that during the infection of various bacteriophages, the increased permeability of the host cell membranes results in partial or complete dissipation of the proton gradient, great reduction of the ATP pool of the cell and severe inhibition of active transport systems (Sabelnikov, 1994). These "symptoms" resemble those observed in *Klebsiella* upon the induction of *kikA*.

It therefore seems more likely that Orf104 is involved in the transport of PRD1 DNA across the membrane, rather than signalling the bound phage to release its DNA.

Figure 4.3 is an interpretation of the presented results in the context of PRD1^s (panel A) as well as KikA⁺ (panel B) phenotypes. Bacteriophages IKe and PRD1 share the same receptor, presumably the pilus tip. Upon the retraction of the pilus, the DNA of the filamentous phage IKe (Figure 4.3 A, left) is pulled through the membrane via a *tolQRAB* transport system (Bradley and Whelan, 1989). The genome of the bacteriophage PRD1 is, however, using a different route. This involves the membrane bound proteinous structure capable of forming a trans-membrane channel (Figure 4.3 A, right, shown as ?). The putative membrane channel can be open, however, only if the Orf104 protein is present. It is speculated here that upon the retraction of the pilus with adsorbed phage (Figure 4.3A a) the phage genome (dsDNA + 5' proteins) passes through the outer membrane into the periplasm (Figure 4.3A b). This can serve as a specific signal which will trigger opening of the channel and will allow the passage of the phage genome accompanied by the proton leakage (Figure 4.3A c). Once inside the cytoplasm, the channel will close, thus restoring the normal function of the membrane. The presence of the membrane channel is only speculative at the present time. However, since the mutations in *kikA* also stimulate conjugation into *Klebsiella*, but not *E. coli*, it is possible that the protein(s) involved in this membrane structure are (partially) encoded by the plasmid *tra* genes and are involved in the conjugal transfer of the plasmid at least into some hosts. It has to be noted that the presence of the Orf70 is not required for the PRD1^s phenotype. The expression of the Orf104 and Orf70 proteins in *Klebsiella*, but not *E. coli*, has a deleterious effect on the cell. It is speculated here that the Orf70 protein may

Figure 4.3. Diagrammatic interpretation of the involvement of the Orf104 and Orf70 proteins in the PRD1^s (panel A) and KikA⁺ (panel B) phenotypes.

Panel A. Following the adsorption of the phage (IKe or PRD1) at the pilus tip, the pilus is retracted and the phage DNA is pulled through the outer membrane. The DNA of the phage IKe is then transported inside the cytoplasm via *tolQRAB* transport system (TolA, left). The genome of the phage PRD1 is too bulky to use the same route. The passage of the genome inside the periplasmic space (a and b) triggers opening of the membrane channel (? , c). The function of this channel is fully dependent on the presence of the Orf104 protein. The PRD1 genome is translocated inside the cytoplasm what is accompanied by a transient proton leakage (c). Once the genome is inside the cytoplasm the channel closes, thus restoring the normal function of the membrane (d).

Panel B. Expression of the Orf104 and Orf70 proteins in *Klebsiella*, but not *E. coli*, has deleterious effect on the cell. The Orf70 may respond to intra- or extra-cellular signal and become phosphorylated (Orf70-P). Modified Orf70-P interacts either by itself (■) or via a putative cytoplasmic protein (Δ) with the Orf104-channel complex causing it to open and remain opened. The induced protein leakage causes dissipation of the membrane potential and eventually the death of the cell.

(OM - outer membrane, IM - inner membrane)

respond to some extra- or intra-cellular signal and become phosphorylated. This modified Orf70-P protein can provide a signal for the putative Orf104-membrane channel complex which cause the opening of this structure. The continuous presence of the Orf70 or the Orf70-P protein, however, prevent this open channel from closing and this leads to the dissipation of the membrane potential and eventually the death of the cell. It is also possible that Orf70 (or Orf70-P) does not interact with the Orf104-channel complex itself. A third, cytoplasmic protein(s) could function as a signal transducer between the Orf70 (or Orf70-P) and the Orf104-channel complex.

Several features of the speculative model described in Figure 4.3 are testable in further genetic experiments. A rather enigmatic observation is that only *Klebsiella*, but not *E. coli* is susceptible to the Kik⁺ phenotype. Since the overexpression of *kikA* in *E. coli* does not cause reduction of viability the reason why *E. coli* is not sensitive to *kikA* cannot be due to the lack of expression of *kikA* proteins. Several possibilities can be listed to explain the resistance of *E. coli* to Kik⁺. The Orf104 and/or Orf70 proteins may be processed differently in *E. coli* than in *Klebsiella*. As indicated in the third part of this thesis (p.108 to p.121), SDS-PAGE analysis of Orf104 protein isolated from both *E. coli* and *Klebsiella* did not disclose any significant differences. Orf104 protein from both species is cleaved at the N-terminus and is exported into periplasm. Also, the Orf104 protein is required for PRD1^s phenotype which can be observed in both *E. coli* and *Klebsiella* suggesting that the Orf104 protein is the same in both species. A similar conclusion, however, cannot be made about the Orf70 protein. This protein was identified only by expressing it from the T7 promoter and labelling it with ³⁵S-methionine in *E. coli*. It is possible, that in a different host such as

Klebsiella it can undergo some modification(s) which does not occur in *E. coli*. As indicated by the computer-assisted search in Prosite database, there are several potential phosphorylation sites in the Orf70 protein. The presence of these sites may indicate that this protein can function as a signal transducer. It is possible, that the signal required for phosphorylation of the Orf70 protein is absent in *E. coli*, thus making it resistant to Kik⁺. As indicated in the hypothetical model of action of the Orf104 and Orf70 proteins in the Kik⁺ phenotype, it is also possible that the Orf70 protein acts *via* another protein(s). It is tempting to speculate that this protein(s) is unique to *Klebsiella* and therefore *E. coli* is not susceptible to Kik⁺ phenotype. The possibility that the target for the *kikA* proteins is missing in *E. coli* must also be considered. However, as indicated by experiments with bacteriophage PRD1, at least one component of the *kikA* region, the Orf104 protein plays the same role in both bacterial species. As proposed in the model for the *kikA* action, the Orf70 protein could act on the membrane complex of Orf104 and a membrane channel. Therefore, the target for the *kikA* proteins, the putative membrane channel should be same in both *E. coli* and *Klebsiella*. Two classes of *Klebsiella* mutants should thus be obtained. One class will render cells resistant to the infection with phage PRD1. This mutation should reside in the gene coding for the putative membrane channel, or one of its components. This mutant should also be resistant to the Kik⁺ phenotype since the mutation will destroy the target for Orf104 protein. However, if the putative membrane channel is part of some more general transport system this class of mutants might not be viable. The other class of mutations will render cells resistant to the Kik⁺ phenotype, but sensitive to the bacteriophage PRD1. These mutations should affect either (i) the hypothetical signal required for modification of the Orf70 protein, (ii) the

cellular proteins involved in the hypothetical modification of the Orf70 protein, or (iii) the hypothetical cellular protein which acts in accord with the Orf70 protein. It was shown here, that the Orf70 protein is not required for the PRD1^s phenotype and therefore none of the above mentioned mutations should affect sensitivity to PRD1. Interestingly, *Klebsiella* cells which survived mating with pCU1 donors are sensitive to PRD1 (Table 4.3) but resistant to the Kik phenotype indicating that they may belong to the second class of mutants. The experiments to characterize this phenomenon are under way in our laboratory. Similar to the chromosomal mutations in *Klebsiella*, mutations in the DNA of pCU1 should be obtained which will abolish the Kik⁻ phenotype but not the PRD1^s phenotype. These mutations are expected to be in the sequence of *orf70* without affecting the amino acid sequence of the Orf104 protein. In addition to conferring the Kik⁻ and PRD1^s phenotypes these mutants should also be transfer proficient in the matings into *Klebsiella* recipients.

References.

- Abee, T., T. R. Klaenhammer, and L. Letellier. 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. *Applied and Environmental Microbiology* 60:1006-1013.
- Akimoto, S., and Y. Ohnishi. 1982. R483 and F plasmid genes promoting RNA degradation: comparative restriction mapping. *Microbiology and Immunology* 26:779-793.
- Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.
- Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research* 31:347-364.
- Anderson, B. L., R. W. Berry, and A. Telser. 1983. A sodium dodecyl sulphate-polyacrylamide gel electrophoresis system that separates peptides and proteins in the molecular weight range of 2500 to 90,000. *Analytical Biochemistry* 132:365-375.
- Austin, S., and A. Abeles. 1983. Partition of unit-copy miniplasmid to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and centromere-like site at which it acts. *Journal of Molecular Biology* 169:373-387.
- Austin, S., M. Ziese, and N. Sternberg. 1981. A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* 25:729-736.
- Bachman, B. J. 1972. Pedigrees of some mutants strains of *Escherichia coli* K-12. *Bacteriological Reviews* 36:525-557.
- Bairoch, A. 1993. The PROSITE dictionary of sites and patterns in proteins, its current status. *Nucleic Acids Research* 21:3097-3103.
- Balzer, D., G. Ziegelin, W. Pansegrau, V. Kruft, and E. Lanka. 1992. KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in region essential for conjugative plasmid transfer. *Nucleic Acids Research* 20:1851-1858.
- Barnford, D. H., and L. Mindich. 1984. Characterization of the DNA-protein complex at the termini of the bacteriophage PRD1 genome. *Journal of Virology* 50:309-315.
- Banerjee, S. K., B. T. Luck, H.-Y. Kim, and V. N. Iyer. 1992. Three clustered origins of replication in a promiscuous plasmid replicon and their differential use in a PolA⁺ strain and a Δ PolA strain of *Escherichia coli* K-12. *Journal of Bacteriology* 174:8139-8143.

- Baquero, F., and F. Moreno. 1984. The microcins. *FEMS Microbiology Letters* 23:117-124.
- Berger, E. A., and L. A. Heppel. 1974. Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino acid permeases of *Escherichia coli*. *The Journal of Biological Chemistry* 249:7747-7755.
- Bernard, P., and M. Couturier. 1992. Cell Killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *Journal of Molecular Biology* 226:735-745.
- Bernard, P., K. Kézdy, L. Van Melderen, J. Steyaert, L. Wyns, M. L. Pato, P. Higgins, and M. Couturier. 1993. The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. *Journal of Molecular Biology* 234:534-541.
- Bibb, M. J., and D. V. Hopwood. 1981. Genetic studies of the fertility plasmid SCP2 and its SCP2⁺ variants in *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* 126:427-442.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7:1513-1523.
- Bishop, L. J., E. S. Bjers, V. L. Davidson, and W. A. Cramer. 1985. Localization of the immunity protein-reactive domain in unmodified and chemically modified COOH-terminal peptides of colicin E1. *Journal of Bacteriology* 164:237-244.
- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles II. A multipurpose cloning system. *Gene* 2:95-113.
- Bourdineaud, J. P., P. Boulanger, C. Lazdunski, and L. Letellier. 1990. *In vivo* properties of colicin A: Channel activity is voltage dependent but translocation may be voltage independent. *Proc. Natl. Acad. Sci. U.S.A.* 87:1037-1041.
- Bowman, C. M., J. E. Dahlberg, T. Ikemura, J. Konisky, and M. Nomura. 1971. Specific inactivation of 16S ribosomal RNA induced by colicin E3 *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 68:964-968.
- Bradley, D. E. 1979. Morphology of pili determined by the N incompatibility group plasmid N3 and interaction with bacteriophages RP4 and IKe. *Plasmid* 2:632-636.
- Bradley, D. E., and J. Whelan. 1989. *Escherichia coli tolQ* mutants are resistant to filamentous bacteriophages that adsorb to the tips, not the shafts, of conjugative pili. *Journal of General Microbiology* 135:1857-1863.

- Braun, V. 1989. The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. *Journal of Bacteriology* 171:6387-6390.
- Braun, V., and C. Hermann. 1993. Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. *Molecular Microbiology* 8:261-268.
- Bravo, A., G. de Torriontegui, and R. Díaz. 1987. Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid. *Molecular and General Genetics* 210:101-110.
- Bravo, A., S. Ortega, G. de Torriontegui, and R. Díaz. 1988. Killing of *Escherichia coli* cells modulated by components of the stability system ParD of plasmid R1. *Molecular and General Genetics* 215:146-151.
- Brosius, J. 1984. Plasmid vectors for the selection of promoters. *Gene* 27:151-160.
- Buck, M., and W. Cannon. 1992. Activator-independent formation of a closed complex between σ^{34} -holoenzyme and *nifH* and *nifU* promoters of *Klebsiella pneumoniae*. *Molecular Microbiology* 6:1625-1630.
- Cardelli, J., and J. Konisky. 1974. Isolation and characterization of an *Escherichia coli* mutant tolerant to colicins Ia and Ib. *Journal of Bacteriology* 119:379-385.
- Cavard, D., A. Bernadac, and C. Lazdunski. 1981. Exclusive localization of colicin A in cell cytoplasm of producing bacteria. *European Journal of Biochemistry* 119:125-131.
- Cavard, D., D. Baty, S. P. Howard, H. M. Verheij, and C. Lazdunski. 1987. Lipoprotein nature of the colicin A lysis protein: Effect of amino acid substitutions at the site of modification and processing. *Journal of Bacteriology* 169:2187-2194.
- Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid, *Journal of Bacteriology* 134:1141-1156.
- Chao, L., and B. Levin. 1981. Structural habitats and evolution of anticompetitors in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 78:6324-6328.

- Chikindas, M. L., M. J. García-Garcera, A. J. M. Driessen, A. M. Ledeboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Applied and Environmental Microbiology* **59**:3577-3584.
- Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to open circular form. *Proc. Natl. Acad. Sci. USA* **62**:1159-1166.
- Cohen, M. L. 1992. Epidemiology of drug resistance: Implications for a post-antimicrobial era. *Science* **257**:1050-1055.
- Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Non-chromosomal antibiotic resistance in bacteria: Genetic transformation in *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110-2114.
- Compton, S. J., and C. G. Jones. 1985. Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry* **151**:369-374.
- Creighton, T. E. 1993. *Proteins: structures and molecular properties*. Second edition. W. H. Freeman and Company. New York
- Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**:17-28.
- Davis, M. A., K. A. Martin, and S. J. Austin. 1992. Biochemical activities of the ParA partition protein of the P1 plasmid. *Molecular Microbiology* **6**:1141-1147.
- Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr. 1980. Carbohydrate transport in bacteria. *Microbiological Reviews* **44**:385-418.
- deGraaf, F. DK., R. J. Planta, and A. H. Stouthamer. 1971. Effect of a bacteriocin produced by *Enterobacter cloacae* on protein synthesis. *Biochimica et Biophysica Acta* **240**:122-136.
- deGraaf, F. K., H. K. D. Niekus, and J. Klotwijk. 1973. Inactivation of bacterial ribosomes *in vivo* and *in vitro* by cloacin DF13. *FEBS Letters* **35**:161-165.
- Dreiseikelmann, B. 1994. Translocation of DNA across bacterial membranes. *Microbiological Reviews* **58**:293-316.

- Duro, A. F., R. Serrano, and C. Asenio. 1979. Effect of the antibiotic microcin 140 on the ATP level and amino acid transport of *Escherichia coli*. *Biochemical and Biophysical Research Communications* 88:297-304.
- Feng, J., K. Yamanaka, H. Niki, T. Ogura, and S. Hiraga. 1994. New killing system controlled by two genes located immediately upstream of the *mukB* gene in *Escherichia coli*. *Molecular and General Genetics* 243:136-147.
- Fields, K. L., and S. E. Luria. 1969a. Effects of colicins E1 and K on transport systems. *Journal of Bacteriology* 97:57-63.
- Fields, K. L., and S. E. Luria. 1969b. Effects of colicins E1 and K on cellular metabolism. *Journal of Bacteriology* 97:64-77.
- Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton. 1982. Broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. *Proc. Natl. Acad. Sci. U.S.A.* 79:1935-1939.
- Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* 48:119-131.
- Garfin, D. E. 1990. One-dimensional gel electrophoresis. *Methods in Enzymology* 182:425-441.
- Gellert, M., K. Mizuuchi, M. O'Dea, and H. Nash. 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proc. Natl. Acad. Sci. U.S.A.* 73:3872-3876.
- Gerdes, K. 1988. The *parB* (*hok/sok*) locus of plasmid R1: a general purpose plasmid stabilization system. *Bio/Technology* 6:1402-1405.
- Gerdes, K., J. E. L. Larsen, and S. Molin. 1985. Stable inheritance of plasmid R1 requires two different loci. *Journal of Bacteriology* 161:292-298.
- Gerdes, K., P. B. Rasmussen, and S. Molin. 1986a. Unique type of plasmid maintenance function: Postsegregational killing of plasmid-free cells. *Proc. Natl. Acad. Sci. USA* 83:3116-3120.
- Gerdes, K., F. W. Bech, S. T. Jørgensen, A. Løbner-Olesen, P. B. Rasmussen, T. Atlung, L. Boe, O. Karlström, S. Molin, and K. von Meyenburg. 1986b. Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon. *The EMBO Journal* 5:2023-2029.

- Gerdes, K., T. Thisted, and J. Martinussen. 1990. Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of a *hok* mRNA species correlated with killing of plasmid-free cells. *Molecular Microbiology* 4:1807-1818.
- Gerdes, K., A. Nielsen, P. Thorsted, and E. G. H. Wagner. 1992. Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable *Hok*, *SrmP* and *PndA* effector messenger RNAs. *Journal of Molecular Biology* 226:637-649.
- Gilchrist, M. J. R., and J. Konisky. 1975. Effect of colicin Ia on transport and respiration in *Escherichia coli*. *The Journal of Biological Chemistry* 250:2457-2462.
- Gill, S. S. 1985. IncN group plasmids and plasmid regions determining the killing of *Klebsiella pneumoniae* and of immunity to killing. M.Sc. Thesis, Carleton University, Ottawa, Ontario, Canada.
- Gill, S. S., and V. N. Iyer. 1982. Nalidixic acid inhibits the conjugal transfer of conjugative N incompatibility group plasmids. *Canadian Journal of Microbiology* 28:256-258.
- Goncharoff, P., S. Saadi, C.-H. Chang, L. H. Saltman, and D. H. Figurski. 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. *Journal of Bacteriology* 173:3463-3477.
- Grewal, K. K. 1990. Plasmid RP4 host-lethal function *kilA* and its repressor *korA* sequences are part of the cryptic tellurite resistance transposon Tn521. *FEMS Microbiology Letters* 69:97-100.
- Gross, J. D. 1963. Cellular damage associated with multiple mating in *E. coli*. *Genetic Research* 4:463-469.
- Guihard, G., H. Bénédicti, M. Besnard, and L. Letellier. 1993. Phosphate efflux through the channels formed by colicins and phage T5 in *Escherichia coli* cells is responsible for the fall in cytoplasmic ATP. *The Journal of Biological Chemistry* 268:17775-17780.
- Hagihira, H., T. H. Wilson, and E. C. C. Lin. 1963. Studies of the glucose-transport system in *Escherichia coli* with α -methylglucoside as substrate. *Biochimica et Biophysica Acta* 78:505-515.
- Hames, B. D. 1981. An Introduction to polyacrylamide gel electrophoresis. In B. D. Hames, and Rickwood, (ed) *Gel Electrophoresis of Proteins: A practical approach*. IRL Press Limited, Oxford and Washington DC.

- Hayes, W. 1965. The genetics of bacteria and their viruses. 2nd Edition, John Wiley and Sons.
- Hengen, P.N. 1991. Molecular cloning, characterization, and nucleotide sequence analysis of *kikA*, a region of the IncN plasmid pCU1 required for killing of *Klebsiella oxytoca*. Ph.D. Thesis, Carleton University, Ottawa, Ontario, Canada.
- Hengen, P. N., D. Denicourt, and V. N. Iyer. 1992. Isolation and characterization of *kikA*, a region on IncN group plasmid that determines killing of *Klebsiella oxytoca*. Journal of Bacteriology 174:3070-3077.
- Higuchi, R. 1990. Recombinant PCR. In Innis et al. (ed) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto.
- Hiraga, S., A. Jaffé, T. Ogura, H. Mori, and H. Takahashi. 1986. F plasmid *cod* mechanism in *Escherichia coli*. Journal of Bacteriology 166:100-104.
- Hiraga, S. 1992. Chromosome and plasmid partition in *Escherichia coli*. Annual Review in Biochemistry 61:283-306.
- Humphreys, G. O., G. A. Willshaw, and E. S. Anderson. 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochemica et Biophysica Acta 383:457-463.
- Hussain, K., K. J. Begg, G. P. C. Salmond, and W. D. Donachie. 1987a. ParD: a new gene coding for a protein required for chromosome partitioning and septum localization in *Escherichia coli*. Molecular Microbiology 1:73-81.
- Hussain, K., E. J. Elliot, and G. P. C. Salmond. 1987b. The ParD mutant of *Escherichia coli* also carries a *gyrA* mutation. The complete sequence of *gyrA*. Molecular Microbiology 1:259-273.
- Inouye, S., A. Nakazawa, and T. Nakazawa. 1987. Expression of the regulatory gene *xytS* on the TOL plasmid is positively controlled by the *xytR* gene product. Proc. Natl. Acad. Sci. USA 84:5182-5186.
- Iyer, V. N. 1989. IncN group plasmids and their genetic systems. In C. M. Thomas (ed) Promiscuous plasmids of Gram negative bacteria. pp.165-183. Academic Press Ltd., London.
- Izard, J. W., and D. A. Kendall. 1994. Signal peptides: exquisitely designated transport promoters. Molecular Microbiology 13:765-773.

- Jacob, F., and E. I. Wollman. 1961. Sexuality and the genetics of bacteria. Academic Press, London.
- Jaffé, A., T. Ogura, and S. Hiraga. 1985. Effects of the *ccd* function of the F plasmid on bacterial growth. *Journal of Bacteriology* 163:841-849.
- Jagura-Burdzy, G., F. Khanim, C. A. Smith, and C. M. Thomas. 1992. Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. *Nucleic Acids Research* 20:3939-3944.
- Jessee, J. 1986. New subcloning efficiency competent cells: $>1 \times 10^6$ transformants/ μ g. Bethesda Research Laboratories FOCUS 8:4.
- Jones, C. P., and S. L. McKnight. 1985. Role of SulB (FtsZ) protein in division inhibition during the SOS response in *Escherichia coli*: FtsZ stabilizes the inhibitor SulA in maxicells. *Proc. Natl. Acad. Sci. U.S.A.* 82:6045-6049.
- Kaback, H.R. 1972. Transport across isolated bacterial cytoplasmic membranes. *Biochimica et Biophysica Acta* 265:367-416.
- Kadner, R.J., and H. H. Winkler. 1975. Energy coupling for methionine transport in *Escherichia coli*. *Journal of Bacteriology* 123:985-991.
- Kato, J., Y. Nishimura, and H. Suzuki. 1989. *Escherichia coli* *parA* is an allele of the *gyrB* gene. *Molecular and General Genetics* 217:178-181.
- Katoaka, M., T. Seki, and T. Yoshida. 1991. Regulation and function of the *Streptomyces* plasmid pSN22 genes involved in pock formation and inviability. *Journal of Bacteriology* 173:7975-7981.
- Kay, R., and J. McPherson. 1987. Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA fragments. *Nucleic Acids Research* 15:2778.
- Kendall, K. J., and S. N. Cohen. 1987. Plasmid transfer in *Streptomyces lividans*: identification of a *kil-kor* system associated with the transfer region of pIJ101. *Journal of Bacteriology* 169:4177-4651.
- Kendall, K. J., and S. N. Cohen. 1988. Complete nucleotide sequence of the *Streptomyces lividans* plasmid pIJ101 and correlation of the sequence with genetic properties. *Journal of Bacteriology* 170:4634-4651.
- Khatoon, H., R. V. Iyer, and V. N. Iyer. 1972. A new filamentous bacteriophage with sex-factor specificity. *Virology* 48:145-155.

- Kim, H.-Y., 1993. Determinants of the *E. coli* polymerase I-independent and polymerase I-dependent pathways of replication of an incompatibility N group plasmid replicon. Ph.D. Thesis, Carleton University, Ottawa, Ontario, Canada.
- Kim, H.-Y., S. K. Banerjee, and V. N. Iyer. 1994. The IncN plasmid replicon: two pathways of DNA polymerase independent replication. *Journal of Bacteriology* 176:7735-7739.
- Kinoshita, N., T. Unemoto, and H. Kobayashi. 1984. Proton motive force is not obligatory for growth of *Escherichia coli*. *Journal of Bacteriology* 160:1974-1077.
- Konarska-Kozłowska, M., and V. N. Iyer. 1981a. The genetic and physical basis of variability in *Escherichia coli* strains carrying a reference IncN group plasmid. *Canadian Journal of Microbiology* 27:616-626.
- Konarska-Kozłowska, M., and V. N. Iyer. 1981b. Physical and genetic organization of the IncN-group plasmid pCU1. *Gene* 14:195-204.
- Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. *Annual Review of Microbiology* 36:125-144.
- Kornacki, J. A., C.-H. Chang, and D. H. Figurski. 1993. *kil-kor* regulon of promiscuous plasmid RK2: Structure, products, and regulation of two operons that constitute the *kilE* locus. *Journal of Bacteriology* 175:5078-5090.
- Kordel, M., and H.-G. Sahl. 1986. Susceptibility of bacterial, eucaryotic and artificial membranes to the disruptive action of the cationic peptides Pep5 and nisin. *FEMS Microbiology Letters* 34:139-144.
- Kotilainen, M. M., A. M. Grahn, J. K. H. Bamford, D. H. Bamford. 1993. Binding of an *Escherichia coli* double-stranded DNA virus PRD1 to a receptor coded by an IncP-type plasmid. *Journal of Bacteriology* 175:3089-3095.
- Kozłowski, M., V. Thatte, P. C. K. Lau, L. P. Visentin, and V. N. Iyer. 1987. Isolation and structure of the replicon of the promiscuous plasmid pCU1. *Gene* 58:217-228.
- Krishnan, B. R., and V. N. Iyer. 1988. Host ranges of the IncN group plasmid pCU1 and its minireplicon in Gram-negative purple bacteria. *Applied and Environmental Microbiology* 54:2273-2276.
- Krishnan, B. R., and V. N. Iyer. 1990. IncN plasmid replicon: A deletion and λ -cloning analysis. *Journal of Molecular Biology* 213:777-788.

- Krishnan, B. R., P. R. Fobert, U. Seitzer, and V. N. Iyer. 1990. Mutations within the replicon of the IncN plasmid pCU1 that affect its *Escherichia coli* *polA*-independence but not its autonomous replication ability. *Gene* **91**:1-7.
- Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss. 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiological Reviews* **53**:367-376.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**:105-132.
- Lagos, R., M. Wilkens, C. Vergara, X. Cecchi, and O. Monasterio. 1993. Microcin E492 forms ion channels in phospholipid bilayer membranes. *FEBS* **321**:145-148.
- Larsen, M. H., and D. H. Figurski. 1994. Structure, Expression, and regulation of the *kilC*' operon of promiscuous IncP α plasmids. *Journal of Bacteriology* **176**:5022-5032.
- Lazdunski, C. J., D. Baty, V. Geli, D. Cavard, J. Morlon, R. Lloubes, S. P. Howard, M. Knibiehler, M. Chartier, S. Varenne, M. Frenette, J.-L. Dasseux, and F. Pattus. 1988. The membrane channel-forming colicin A: synthesis, secretion, structure, action and immunity. *Biochimica et Biophysica Acta* **947**:445-464.
- Lehnherr, H., E. Maguin, S. Jafri, and M. B. Yarmolinsky. 1993. Plasmid addiction genes of bacteriophage P1: *doc*, which causes cell death on curing of prophage, and *phd*, which prevents host death when prophage is retained. *Journal of Molecular Biology* **233**:414-428.
- Lehnherr, H., and M. B. Yarmolinsky. 1994. The *phd-doc* addiction module of plasmid prophage P1. International meeting on Plasmid Biology '94, August 25 - 30, Banff, Alberta, Canada.
- Letellier, L. 1992. In vivo properties of colicin A: channel activity and translocation across the envelope of *Escherichia coli*. *Biochimica et Biophysica Acta* **1101**:218-220.
- Levengood-Freyermuth, S. K., E. M. Click, and R. E. Webster. 1993. Role of carboxy-terminal domain of TolA in protein import and integrity of the outer membrane. *Journal of Bacteriology* **175**:222-228.
- Loh, S. M., D. S. Cram, and R. A. Skurray. 1988. Nucleotide sequence and transcriptional analysis of a third function (Flm) involved in F-plasmid maintenance. *Gene* **66**:259-268.

- Luckey, M., and H. Nikaido. 1980. Specificity of diffusion channels produced by lambda phage receptor protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 77:167-171.
- Lyra, C., H. Savilahti, and D. H. Bamford. 1991. High-frequency transfer of linear DNA containing 5'-covalently linked terminal protein: electroporation of bacteriophage PRD1 genome into *Escherichia coli*. *Molecular and General Genetics* 228:65-69.
- MacNeil, D., J. Zhu, and W. J. Brill. 1981. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: Isolation and characterization of strains with *nif-lac* fusions. *Journal of Bacteriology* 145:348-357.
- Maki, S., S. Takiguchi, T. Miki, and T. Horiuchi. 1992. Modulation of DNA supercoiling activity of *Escherichia coli* DNA gyrase by F plasmid proteins: Antagonistic actions of LetA (CcdA) and LetD (CcdB) proteins. *The Journal of Biological Chemistry* 267:12244-12251.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.
- Manoil, C., and J. Beckwith. 1985. *TnphoA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. U.S.A.* 82:8129-8133.
- Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo. 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for the stable maintenance of plasmid R100. *Journal of Bacteriology* 175:6850-6856.
- McCann, J., N. E. Spingarn, J. Kabori, and B. N. Ames. 1975. Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. U.S.A.* 72:979-983.
- Merrick, M. J. 1993. In a class of its own - the RNA polymerase sigma factor σ^{54} (σ^N). *Molecular Microbiology* 10:903-909.
- Miki, T., J. A. Park, K. Nagao, N. Murayama, and T. Horiuchi. 1992. Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*. Mutants of DNA gyrase subunit A suppress *letD* (*ccdB*) product growth inhibition. *Journal of Molecular Biology* 225:39-52.
- Miller, C. A., S. L. Beaucage, and S. N. Cohen. 1990. Role of DNA superhelicity in partitioning of the pSC101 plasmid. *Cell* 62:127-133.

- Minchin, S. D., S. Austin, and R. A. Dixon. 1988. The role of activator binding sites in transcriptional control of the divergently transcribed *nifF* and *nifH* promoters from *Klebsiella pneumoniae*. *Molecular Microbiology* 2:433-442.
- Moré, M. I., and S. C. Winans. 1994. Two pKM101 encoded genes are required for repression of conjugal transfer operons. International meeting on Plasmid Biology '94, August 25 - 30, Banff, Alberta, Canada.
- Morlon, J., R. Lloubes, S. Varenne, M. Chartier, and C. Lazdunski. 1983. Complete nucleotide sequence of the structural gene for colicin A, a gene translated at non-uniform Rate. *Journal of Molecular Biology* 170:271-285.
- Morlon, J., M. Chartier, M. Bidaud, and C. Lazdunski. 1988. The complete nucleotide sequence of the colicinogenic plasmid ColA. High extent of homology with ColE1. *Molecular and General Genetics* 211:231-243.
- Motallebi-Veshareh, M., D. Balzer, E. Lanka, G. Jagura-Burdzy, and C. M. Thomas. 1992. Conjugative transfer functions of broad-host-range plasmid RK2 are coregulated with vegetative replication. *Molecular Microbiology* 6:907-920.
- Nagel De Zwaig, R., and S. E. Luria. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. *Journal of Bacteriology* 94:1112-1123.
- Nielsen, A. K., P. Thorsted, T. Thisted, E. G. H. Wagner, and K. Gerdes. 1991. The rifampicin-inducible genes *srnB* from F and *pnd* from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. *Molecular Microbiology* 5:1961-1973.
- Ninfa, A. J., and B. Magasanik. 1986. Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 83:5909-5913.
- Nomura, M. 1963. Mode of action of colicins. Cold Spring Harbour Symposium in Quant. Biology 28:315-324.
- Nordström, K. and S. J. Austin. 1989. Mechanisms that contribute to the stable segregation of plasmids. *Annual Review of Genetics* 23:37-69.
- North, A. K., K. E. Klose, K. M. Stedman, and S. Kustu. 1993. Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. *Journal of Bacteriology* 175:4267-4273.

- Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, S. Falkow. 1976. Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriological Reviews* 40:168-189.
- Ogura, T., and S. Hiraga. 1983a. Partition mechanism of F plasmid: Two plasmid gene-encoded products and a cis-acting region are involved in partition. *Cell* 32:351-360.
- Ogura, T., and S. Hiraga. 1983b. Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc. Natl. Acad. Sci. U.S.A.* 80:4784-4788.
- Ohnishi, Y. 1975. F factor promotes turnover of stable RNA in *Escherichia coli*. *Science* 187:257-258.
- Ohnishi, Y., and S. Akimoto. 1980. I-like R-plasmids promote degradation of stable ribonucleic acid in *Escherichia coli*. *Journal of Bacteriology* 144:833-835.
- Ohno, S., and K. Imahori. 1978. Colicin E3 is an endonuclease. *Journal of Biochemistry* 84:1637-1640.
- Olsen, R. H., J.-S. Siak, and R. H. Gray. 1974. Characteristics of PRD1, a plasmid-dependent broad host range DNA bacteriophage. *Journal of Virology* 14:689-699.
- Olsen, R. H., J.-S. Siak, and P. L. Shipley. 1977. Plasmid RP1-encoded surface components: a somatic receptor for phage PRD1. in D. Schlessinger (ed), *Microbiology-1977*, American Society for Microbiology, Washington, pp.138-144.
- Orr, E., N. F. Fairweather, I. B. Holland, and R. H. Pritchard. 1979. Isolation and characterization of a strain carrying a conditional lethal mutation in the *cou* gene of *Escherichia coli* K12. *Molecular and General Genetics* 177:103-112.
- Ou, T. J. 1980. Role of surface exclusion genes in lethal zygotis in *Escherichia coli* K-12 mating. *Molecular and General Genetics* 180:165-175.
- Papp, P. P., and V. N. Iyer. 1995. Determination of the binding sites of RepA, a replication initiator protein of the basic replicon of the IncN group plasmid pCU1. *Journal of Molecular Biology* 246: 595-608.
- Paterson, E. S., and V. N. Iyer. 1992. The *oriT* region of the conjugative transfer system of plasmid pCU1 and specificity between it and the *mob* region of other N *tra* plasmids. *Journal of Bacteriology* 174:499-507.

- Pattus, F., F. Heitz, C. Martinez, S. W. Provencher, and C. Lazdunski. 1985. Secondary structure of the pore-forming colicin A and its C-terminal fragment. Experimental fact and structure prediction.. *European Journal of Biochemistry* 152:681-689.
- Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* 85:2444-2448.
- Piperno, J. R., and D. L. Oxender. 1968. Amino acid transport systems in *Escherichia coli* K12. *The Journal of Biological Chemistry* 243:5914-5920.
- Pohlman, R. F., H. D. Genetti, and S. C. Winans. 1994. Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. *Molecular Microbiology* 14:655-668.
- Postle, K. 1990. TonB and the Gram-negative dilemma. *Molecular Microbiology* 4:2019-2025.
- Poulsen, L. K., N. W. Larsen, S. Molin, and P. Andersson. 1989. A family of genes encoding a cell-killing function may be conserved in all Gram-negative bacteria. *Molecular Microbiology* 3:1463-1472.
- Poulsen, L. K., A. Refn, S. Molin, and P. Andersson. 1991a. Topographic analysis of the toxic Gef protein from *Escherichia coli*. *Molecular Microbiology* 5:1627-1637.
- Poulsen, L. K., A. Refn, S. Molin, and P. Andersson. 1991b. The *gef* gene from *Escherichia coli* is regulated at the level of translation. *Molecular Microbiology* 5:1639-1648.
- Poulsen, L. K., N. W. Larsen, S. Molin, and P. Andersson. 1992. Analysis of an *Escherichia coli* mutant strain resistant to the cell-killing function encoded by the *gef* gene family. *Molecular Microbiology* 6:895-905.
- Prentki, P., and H. M. Krisch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29:303-313.
- Prince, J. 1994. The effect of RepA and the group I iterons on the electrotransformation of *Klebsiella oxytoca* UN2979 with plasmids containing the replicon region of plasmid pCU1. B.Sc. Thesis, Carleton University, Ottawa, Ontario, Canada.
- Rasmussen, P. B., K. Gerdes, and S. Molin. 1987. Genetic analysis of the *parB'* locus of plasmid R1. *Molecular and General Genetics* 209:122-128.

- Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* 113:237-251.
- Riley, M. A. 1993. Positive selection for colicin diversity in bacteria. *Molecular Biology and Evolution* 10:1048-1059.
- Riley, M. A., and D. M. Gordon. 1992. A survey of col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of col-plasmid lineages. *Journal of General Microbiology* 138:1345-1352.
- Rodríguez, M. 1981. Killing of *Klebsiella pneumoniae* promoted by IncN group and closely related plasmids. M.Sc. Thesis. Carleton University, Ottawa, Ontario, Canada.
- Rodríguez de González, M. 1994. Identification and properties of a region on plasmid pCU1 needed for the establishment in *Klebsiella oxytoca* of immunity to killing by the same plasmid. Ph.D. Thesis, Carleton University, Ottawa, Ontario, Canada.
- Rodríguez, M., and V. N. Iyer. 1981. Killing of *Klebsiella pneumoniae* mediated by conjugation with bacteria carrying antibiotic resistance plasmids of the group N. *Plasmid* 6:141-147.
- Rosenberg, A. H., B. N. Lade, D. Chui, S. Lin, J. J. Dunn, and F. W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56:125-135.
- Rotheim, M. B., B. Love, V. Thatte, and V. N. Iyer. 1988. A demonstration that pCU1 *tra* gene products are not required in the killing of *Klebsiella pneumoniae*. *Plasmid* 19:161-163.
- Ruiz-Echevarría, M. J., G. de Torrontegui, G. Giménez-Gallego, and R. Díaz-Orejas. 1991a. Structural and functional comparison between the stability systems ParD of plasmid R1 and Ccd of plasmid F. *Molecular and General Genetics* 225:355-362.
- Ruiz-Echevarría, M. J., A. Berzal-Herranz, K. Gerdes, and R. Díaz-Orejas. 1991b. The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. *Molecular Microbiology* 5:2685-2693.
- Sabelnikov, A. G. 1994. Nucleic acid transfer through cell membranes: towards the underlying mechanisms. *Prog. Biophys. molec. Biol.* 62:119-152.

- Salmon, M. A., L. Van Melder, P. Bernard, and M. Couturier. 1994. The antidote and autoregulatory functions of the F plasmid CcdA protein: a genetic and biochemical survey. *Molecular and General Genetics* 244:530-538.
- Saltman, L. H., K.-S. Kim, and D. H. Figurski. 1991. The *kilA* operon of promiscuous plasmid RK2: the use of a transducing phage (λ pklaA-1) to determine the effect of the lethal *klaA* gene on *Escherichia coli* cells. *Molecular Microbiology* 5:2673-2683.
- Sander, C., and R. Schneider. 1991. Database of homology-derived structures and the structural meaning of sequence alignment. *Proteins* 9:56-68.
- Sanger, F., S. Nickel, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467.
- Schaffer, H. E., and R. R. Sederoff. 1981. Improved estimation of DNA fragments length from agarose gels. *Anal. Biochem.* 115:113-122.
- Schein, S. J., B. L. Kagan, and A. Finkelstein. 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature* 276:159-163.
- Schriner, H. C., D. H. Bechhofer, R. F. Pohlman, C. Young, P. A. Borden, and D. H. Figurski. 1985. Replication control of promiscuous plasmid RK2: *kil* and *kor* functions affect expression of the essential replication gene *trfA*. *Journal of Bacteriology* 163:228-237.
- Seifter, S., and S. England. 1990. Analysis for protein modifications and nonprotein cofactors. *Methods in Enzymology* 182:626-646.
- Selvaraj, G., and V. N. Iyer. 1984. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. *Journal of Bacteriology* 156:1292-1300.
- Simoni, R. D., and P. W. Postma. 1975. The energetics of bacterial active transport. *Annual Review in Biochemistry* 44:523-554.
- Skurray, R. A., and P. Reeves. 1973. Physiology of *Escherichia coli* K-12 during conjugation: altered recipient cell functions associated with lethal zygosis. *Journal of Bacteriology* 114:11-17.
- Smith, H. W. 1974. A search for transmissible pathogenic characteristics in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled lethal character closely associated, or identical, with colicin V. *Journal of General Microbiology* 83:95-111.

- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98:503-517.
- Stein, D. S., K. J. Kendall, and S. N. Cohen. 1989. Identification and analysis of transcriptional regulatory signals for the *kil* and *kor* loci of *Streptomyces* plasmid pIJ101. *Journal of Bacteriology* 171:5768-5775.
- Stout, V., A. S. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman. 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. *Journal of Bacteriology* 173:1738-1747.
- Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* 189:113-130.
- Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology* 185:60-89.
- Summers, D. K., and D. J. Sherratt. 1984. Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. *Cell* 36:1097-1103.
- Sun, T.-P., and R. E. Webster. 1986. *fii*, a bacterial locus required for filamentous phage infection and its relation to colicin-tolerant *tolA* and *tolB*. *Journal of Bacteriology* 165:107-115.
- Sun, T.-P., and R. E. Webster. 1987. Nucleotide sequence of a gene cluster involved in entry of E colicins and single-stranded DNA of infecting filamentous bacteriophages into *Escherichia coli*. *Journal of Bacteriology* 169:2667-2674.
- Tam, J. E., and B. C. Kline. 1989a. Control of the *ccd* operon in plasmid F. *Journal of Bacteriology* 171:2353-2360.
- Tam, J. E., and B. C. Kline. 1989b. The F plasmid *ccd* autorepressor is a complex of CcdA and CcdB proteins. *Molecular and General Genetics* 219:26-32.
- Thatte, V. 1984. The N transfer system of bacterial conjugation: genetic analysis and relationship to phage sensitivity and the killing of *Klebsiella pneumoniae*. Ph.D. Thesis. Carleton University, Ottawa, Ontario, Canada.
- Thatte, V., S. Gill, and V. N. Iyer. 1985a. Regions on plasmid pCU1 required for the killing of *Klebsiella pneumoniae*. *Journal of Bacteriology* 163:1296-1299.

- Thatte, V., D. E. Bradley, and V. N. Iyer. 1985b. N conjugative transfer system of plasmid pCU1. *Journal of Bacteriology* 163:1229-1236.
- Thisted, T., and K. Gerdes. 1992. Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1. Sok antisense RNA regulates *hok* gene expression indirectly through the overlapping *mok* gene. *Journal of Molecular Biology* 223:41-54.
- Thisted, T., A. K. Nielsen, and K. Gerdes. 1994a. Mechanism of post-segregational killing: translation of Hok, SrmB and Pnd mRNAs of plasmids R1, F and R483 is activated by 3' -end processing. *The EMBO Journal* 13:1950-1959.
- Thisted, T., N. S. Sørensen, E. G. H. Wagner, and K. Gerdes. 1994b. Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 5' -end single-stranded leader and competes with the 3' -end of Hok mRNA for binding to the *mok* translational initiation region. *The EMBO Journal* 13:1960-1968.
- Thomson, V. J., O. S. Jovanovic, R. F. Pohlman, C.-H. Chang, and D. H. Figurski. 1993. Structure, function, and regulation of the *kilB* locus of promiscuous plasmid RK2. *Journal of Bacteriology* 175:2423-2435.
- Traub, I., and V. Braun. 1994. Energy-coupled colicin transport through the outer membrane of *Escherichia coli* K-12: mutated TonB proteins alter receptor activities and colicin uptake. *FEMS Microbiology Letters* 119:65-70.
- Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo. 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. *Journal of Bacteriology* 170:1461-1466.
- Tsuchimoto, S., Y. Nishimura, and E. Ohtsubo. 1992. The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *Journal of Bacteriology* 174:4205-4211.
- Turner, R. J., J. H. Weiner, and D. E. Taylor. 1994. Characterization of the growth inhibition phenotype of the *kilAtelAB* operon from the IncP α plasmid RK2Te'. *Biochem. Cell Biology* 72:333-342.
- Van Melder, L., P. Bernard, and M. Couturier. 1994. Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Molecular Microbiology* 11:1151-1157.
- von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Research* 14:4683-4690.

- von Meyenburg, K., B. B. Jørgensen, O. Michelson, L. Sørensen, and J. E. G. McCarthy. 1985. Proton conduction by subunit α of the membrane-bound ATP synthase of *Escherichia coli* revealed after induced overproduction. *The EMBO Journal* 4:2357-2363.
- Watson, R. J. 1972. Expression of the Rel gene 7 during R17 phage infection. M.Sc. Thesis, Carleton University, Ottawa, Canada.
- Weaver, C. A., B. L. Kagan, A. Finkelstein, and J. Konisky. 1981. Mode of action of colicin Ib. Formation of ion-permeable channels. *Biochimica et Biophysica Acta* 645:137-142.
- Williams, D. R., and C. M. Thomas. 1992. Active partitioning of bacterial plasmids. *Journal of General Microbiology* 138:1-16.
- Williams, D. R., M. Motallebi-Veshareh, and C. M. Thomas. 1993. Multifunctional repressor KorB can block transcription by preventing isomerization of RNA polymerase-promoter complexes. *Nucleic Acids Research* 21:1141-1148.
- Wilmes-Reisenberg, M., and B. L. Wanner. 1992. *TnphoA* and *TnphoA'* elements for making and switching fusions for study of transcription, translation and cell surface localization. *Journal of Bacteriology* 174:4558-4575.
- Winans, S. C., and G. C. Walker. 1985. Identification of pKM101-encoded loci specifying potentially lethal gene products. *Journal of Bacteriology* 161:417-424.
- Windle, B.E. 1986. Phage lambda and plasmid expression vectors with multiple cloning sites and *lacZ* α -complementation. *Gene* 45:95-99.
- Wood, P. M. 1978. Periplasmic location of the terminal reductase in nitrate respiration. *FEBS Letters* 92:214-218.
- Yang, C.-C., and J. Konisky. 1984. Colicin V treated *Escherichia coli* does not generate membrane potential. *Journal of Bacteriology* 158:757-759.
- Yocum, R. 1989. Proposed amendment of the NIH guidelines regarding *Klebsiella oxytoca*. *Federal Register (U.S.A.)* 54:36703-36704.
- Young, C., A. S. Prince, and D. H. Figurski. 1985. *korA* function of promiscuous plasmid RK2: an autorepressor that inhibits expression of host-lethal gene *kilA* and replication gene *trfA*. *Proc. Natl. Acad. Sci. USA* 82:7374-7378.

- Zaman, S., H. Richards, and J. Ward. 1992. Expression and characterization of the *korB* gene product from the *Streptomyces lividans* plasmid pIJ101 in *Escherichia coli* and determination of its binding site on the *korB* and *kilB* promoters. *Nucleic Acids Research* 20:3693-3700.

END

1 3-1 1-9 5

FIN