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IN VIVO INVESTIGATIONS OF GENETICALLY MODIFIED MICROORGANISMS USING GERM-FREE RATS

Ph.D. Thesis
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Abstract: Risk evaluation of genetically modified microorganism (GMMO) in relation to human health effects brings into consideration the ability of the microorganism to survive and colonise the gastrointestinal tract and the potential gene transfer to the resident microbiota. Different biological containment systems based on the activation of a killing gene have resulted in the reduced survival of the contained *Escherichia coli* or in the prevention of plasmid transfer between *E. coli* in the gnotobiotic rat. Gene transfer between *Lactococcus lactis* strains has been demonstrated in the gastrointestinal tract of gnotobiotic rats. The plasmid pLMP1 containing a selectable marker of a *L. lactis* strain was not transferred. The use of germ-free rats has led to a reduction in the number of laboratory animals needed for obtaining information regarding the fate and effect of GMMO in the mammalian gastrointestinal tract.

Keywords: Germ-free, gnotobiotic, biological containment, genetically modified microorganisms, *Escherichia coli*, *Lactococcus lactis*.

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CONTENTS:

PREFACE 5

SUMMARY 6

DANSK RESUMÉ 8

LIST OF PRESENTED PAPERS : 10

1. INTRODUCTION 11

2. RISK ASSESSMENT 13

3. THE MAMMALIAN GASTROINTESTINAL TRACT 17

4. MODELS OF THE MAMMALIAN GASTROINTESTINAL TRACT 20

5. BIOLOGICAL CONTAINMENT 24

6. STUDY OF SURVIVAL AND COLONISATION OF THE GASTROINTESTINAL TRACT 27

7. STUDY OF GENE TRANSFER IN THE GASTROINTESTINAL TRACT USING GERM-FREE RATS 31

8. STUDY OF ALTERNATIVE MARKERS USING GERM-FREE RATS 35

9. STUDY OF BIOLOGICAL CONTAINMENT SYSTEMS AND ALTERNATIVE MARKERS USING ANTIBIOTIC TREATED CONVENTIONAL AND CONVENTIONAL RATS 41

10. EVALUATION AND PERSPECTIVES 45

REFERENCES: 50

APPENDIX A: STUDY OF A BIOLOGICAL CONTAINMENT SYSTEM IN CONVENTIONAL, NORFLOXACIN TREATED CONVENTIONAL AND GERM-FREE RATS. 61

APPENDIX B: SHORT INTRODUCTION TO GNOTOBIOLOGY 71

APPENDIX C: GLOSSARY 72

PRESENTED PAPERS: 73

PREFACE

This Ph.D Thesis has been submitted to the University of Roskilde, Institute of Life Sciences and Chemistry. Supervisors have been Dr. Scient Gustaw Kerszman, University of Roskilde and D.V.M. Otto Meyer, National Food Agency of Denmark.

The studies reported here were done as part of my work at the National Food Agency of Denmark, Institute of Toxicology. Part of the studies has been financed by the Danish biotechnology programme through the Danish Microbiology Centre.

I would like to thank all at the Institute of Toxicology for a good social and intellectually inspiring atmosphere. Especially, I would like to thank Jørgen Schlundt and Grethe Fischer for their effort and support during many discussions and practical achievements, Otto Meyer for useful discussions in animal science and Lejf Burkal together with the animal technicians for the practical implementation of many ideas.

Finally I would like to show my gratitude to Prof. T. Midtvedt and Docent Elisabeth Norin, who ever since my first day as a gnotobiologist at the International Symposium on Gnotobiology in Versailles, have been intellectually stimulating and encouraging.

To Nils, Magnus and Rasmus

SUMMARY

The ability of genetically modified microorganisms (GMMO) to survive and the ability of gene transfer in the environment are important parameters in risk assessment. Limitations in survival and gene transfer are the key elements of biological containment. Molecular constructions have been made to improve the detection and biological containment of GMMO. The use of *Escherichia coli* and *Lactococcus lactis* as test microorganisms in various gastrointestinal models has enabled the study of microbial detection, survival, colonisation and gene transfer between microorganisms. The models of the mammalian gastrointestinal tract exhibit different degrees of colonisation resistance ranging from germ-free rats with no detectable microbiota to conventional rats with a normal protective microbiota.

A biological containment system resulting in bacterial suicide has been tested using germ-free rats and wildtype *E. coli* as test microorganism.The suicide system is based on random activation of the killing genes *hok* or *gef*. The effect of *hok* or *gef* gene was observed as reduced survival of the contained *E. coli* in situation of competition with an existing *E. coli* wildtype population in gnotobiotic rats. This experimental design was simulating an exposure of the "normal" *E. coli* population to genetically modified *E. coli*. In monoassociated gnotobiotic rats the suicide gene containing *E. coli* did not die out. This may have been expected from the results of earlier *in vitro* experiments.
- Conclusion: It is possible to demonstrate an effect of the biological containment systems on survival of *E. coli* using gnotobiotic rat.

A biological containment system based on the activation of a killing gene *rel*F in *E. coli* has also been tested using the germ-free rat. This biological containment system was constructed to prevent transfer of plasmids to wildtype bacteria. No transfer was observed in the experiment using the contained plasmid.

The use of germ-free rats for the study of microorganisms is not restricted to test microorganisms considered natural inhabitants of the gastrointestine. Gene transfer between different strains of *Lactococcus lactis* has also been demonstrated using gnotobiotic rats.
- Conclusion: It is possible to demonstrate plasmid transfer or lack of transfer between related bacteria in the gastrointestinal tract of mammals using gnotobiotic rats.

Genes coding for resistance to different antibiotics are well-known markers used in GMMO. As an alternative to the antibiotic resistance marker a nonselectable marker based on the identification of a specific altered nucleotide sequence has been developed. The identification of such a marker involves the application of molecular techniques like specific probe hybridization and the polymerase chain reaction (PCR). From experiments

using this silent mutation as a marker in a *Lactococcus lactis* strain it was concluded, that the plasmid carrying the silent mutation was not transferred, and thus was suitable as a strain marker.
- Conclusion: The gnotobiotic rat provided optimal conditions for the study of gene transfer and for the comparison of phenotypic detection with methods of nucleic acid detection. The results obtained suggest that the method of detection by an antibiotic resistance marker may be considered more useful than the PCR based method.

Norfloxacin treatment of conventional rats has proven useful for a reduction of the colonisation resistance of the rat by temporal elimination of Enterobacteriaceae. The results from experiments with biologically contained *E. coli* in the norfloxacin treated rats supports the previous made statement based on the experiments in the gnotobiotic rat: The competition between the *gef* gene containing *E. coli* and a closely related species of a resident microbiota is the most important factor leading to the exclusion of *E. coli* with the *gef* gene. When the same biologically contained *E. coli* was given to conventional rats, it was eliminated rapidly within few days from the gastrointestinal tract. Elimination of strains was similar irrespective of their content of the *gef* gene. Dosing conventional rats with *Lactococcus lactis* containing the transferable plasmid, pAMß1 resulted in lateral transfer of the plasmid to an Enterococcus from the microbiota.
- Conclusion: Norfloxacin treated and conventional rats are useful models in the elucidation of the interaction of the GMMO with the normal microbiota.

The successful use of germ-free rats resulted in information regarding the survival and colonisation of GMMO as well as gene transfer between GMMO and microorganisms in the mammalian gastrointestinal tract. Some of this information would have been impossible or difficult to obtain using conventional rats. The use of germ-free rats has resulted in a significant reduction of the number of laboratory animals necessary to obtain this information. In the future the effect of microorganisms (including GMMO) will be in focus in relation to risk assessment, including potential risk and potential benefit. The germ-free rat and the human faecal flora associated rat will be an obvious choice of these studies.

DANSK RESUMÉ

I en risikovurdering af genetisk modificerede mikroorganismer (GMMO) indgår oplysning om mikroorganismens evne til at overleve og evnen til at videregive genetisk information til andre organismer i miljøet. Begrænsning af evne til overlevelse og genoverførsel indgår i begrebet biologisk indeslutning. Med henblik på at forbedre genfindelse og den biologiske indeslutning har man udviklet specielle molekylærbiologiske konstruktioner til anvendelse i GMMO. Brug af *Escherichia coli* og *Lactococcus lactis* som testmikrorganismer i forskellige mave-tarm modeller har muliggjort undersøgelse af genfindelse, overlevelse, kolonisation samt genoverførsel mellem mikroorganismer. Mave-tarm modellerne udviser forskellig modstandsdygtighed overfor kolonisation af udefra kommende mikroorganismer (kolonisationsresistens). Modellerne spænder fra kim-fri rotte, som ikke har nogen påviselig mikroflora til konventionelle rotter med en normal beskyttende tarmflora.

Et biologisk indeslutningssystem baseret på en tilfældig aktivering af såkaldte dræber gener, *hok* eller *gef* er blevet undersøgt *in vivo*. I undersøgelsen af dette "selvmordssystem" indgik et vildtype isolat af *E. coli* som testbakterie og mave-tarm modellen kim-fri rotte. Undersøgelsen var designet så den efterlignede naturlig eksponering af en eksisterende *E. coli* population for genetisk modificerede *E. coli*. Effekten af det aktiverede *hok* eller *gef* gen kunne ses som en reduceret overlevelse af den indesluttede *E. coli*, når denne var i konkurrence med den tilstedeværende population af *E. coli* i gnotobiotiske rotter. I gnotobiotiske rotter, hvori der alene findes biologisk indesluttede *E.coli*, uddør disse *E. coli* ikke. Dette kunne man have forventet ud fra *in vitro* forsøg.
- Konklusion: Ved at anvende gnotobiotiske rotte er det muligt at påvise det biologiske indeslutnings systems effekt på overlevelsen af *E. coli*.

Et biologisk indeslutningssystem baseret på aktivering af dræber genet *rel*B har ligeledes været undersøgt i gnotobiotiske rotter. Dette biologiske indeslutningssystem har til formål at forhindre overførsel af plasmider fra genetisk modificerede mikroorganismer til bakterier i miljøet. I undersøgelsen kunne der ikke iagttages overførsel af det indesluttede plasmid.

Anvendelsen af kim-fri rotte til undersøgelse af mikroorganismer, er ikke begrænset til kun at omfatte undersøgelse af mikroorganismer, der er hjemmehørende i mave-tarmkanalen. Genoverførsel mellem forskellig stammer af *Lactococcus lactis* er blevet påvist i gnotobiotiske rotter.
- Konklusion: Det er muligt at vise plasmidoverførsel eller manglende overførsel ved at anvende gnotobiotiske rotter.

Gener, som koder for modstandsdygtighed overfor forskellige antibiotika anvendes ofte som markører i GMMO. Som et alternativ til sådanne markører er der udviklet en markør, der beror på genfindelse af en specifik

ændret nucleotidsekvens. I dette tilfælde drejer det sig om en såkaldt tavs mutation. Markørens tilstedeværelse kan påvises ved hybridisering med en specifik probe og polymerase kæde reaktion teknikken (PCR). Ud fra undersøgelser med en *L. lactis* stamme med denne plasmidbårne markør, kunne det konstateres, at plasmidet med den tavse mutation ikke kunne overføres, og derfor ville være anvendelig som stamme markør.
- Konklusion: Ved at anvende gnotobiotiske rotter fås optimale betingelser for undersøgelse af genoverførsel og for sammenligning af genfindelse ud fra fænotype henholdsvis genotype. Ud fra de opnåede resultater er det umiddelbare indtryk, at den traditionelle genfindelsesmetode med brug af antibiotika resistens markører er mere anvendelig end metoden baseret på PCR.

Behandling af konventionelle rotter med antibiotikummet Norfloxacin bevirker nedsat modstandsdygtighed overfor kolonisation, idet Enterobacteriaceae elimineres. Resultaterne fra undersøgelser af biologisk indesluttede *E. coli* i norfloxacinbehandlede rotter støtter antagelsen, udledt af tidligere undersøgelser i gnotobiotiske rotter: Konkurrence mellem *E. coli* indeholdende *gef* genet og en nært beslægtet art fra den etablerede tarmflora er den vigtigste faktor, der fører til udvaskning af den indesluttede *E. coli*. Når den samme biologisk indesluttede *E. coli* gives til konventionelle rotter udvaskes den fra tarmen indenfor få dage. Yderligere undersøgelser viser, at en sådan udvaskning forekommer uanset om *gef* genet er tilstede eller ej i de undersøgte *E. coli*.
Da *L. lactis* indeholdende et overførbart plasmid pAMß1 blev givet til konventionelle rotter kunne der iagttages en overførsel af plasmidet til Enterococcus i tarmfloraen.
- Konklusion: Norfloxacinbehandlede og konventionelle rotter er nyttige modeller til belysning af samspillet mellem GMMO og den normale tarmflora.

Den vellykkede anvendelse af kim-fri rotte har ført til viden omkring GMMO overlevelse og kolonisation samt genoverførsel mellem GMMO og mikroorganismer i pattedyrs tarm. En del af viden ville have været vanskelig eller umulig at opnå ved at anvende konventionelle rotter. Brugen af kim-fri rotte har endvidere bevirket reduktion i antallet af forsøgsdyr for at opnå denne viden. I fremtiden vil der være fokus på mikroorganismers (inklusive GMMO) effekt på menneskers sundhed. Den sundhedsmæssige vurdering vil ikke blot omhandle eventuelle risici; men også en eventuel gavnlig effekt af mikroorganismen. Den kim-fri rotte og en rotte, hvor rottetarmfloraen er erstattet med menneskets tarmflora vil være gode modeller for undersøgelse af specifikke mikroorganismers effekt.

LIST OF PRESENTED PAPERS :

Paper I: *Jacobsen, B.L., Schlundt J. & Fischer G. (1993)*: Study of a Conditional Suicide System for Biological Containment of Bacteria in Germ-free Rats. Microbial Ecology in Health and Disease 6:109-118.

Paper II: *Jacobsen B.L., Schlundt J. & Fischer G. (1994)*: The Use of Germ-free Rats for the Study of Fate and Effect of Genetically Modified Microorganisms. Submitted to Microecology and Therapy.

Paper III: *Knudsen S., Saadbye P., Hansen L.H., Collier A., Jacobsen B.L., Schlundt J. & Karlström O.H. (1995)*: Development and Testing of Improved Suicide Functions for Biological containment of Bacteria. Applied and Environmental Microbiology 61:985-991.

Paper IV: *Schlundt J., Saadbye P., Lohmann B., Jacobsen B.L. & Nielsen E.M. (1994)*: Conjugal Transfer of Plasmid DNA between *Lactococcus lactis* Strains and the Distribution of Transconjugants in the Digestive Tract of Gnotobiotic Rats. Microbial Ecology in Health and Disease 7:59-69.

Paper V: *Brockmann E., Jacobsen B.L., Hertel C., Ludwig W. & Schleifer K.H. (1995)*: Monitoring of Genetically Modified *Lactococcus lactis* in Gnotobiotic and Conventional Rats by Using Antibiotic Resistance Markers and Specific Probe or Primer Based Methods. Submitted to Systematic and Applied Microbiology.

1. INTRODUCTION

The rapid developments in recombinant DNA technology have been followed by widespread use of genetically modified organisms (GMO), including microorganisms (GMMO). The molecular techniques have provided tools to a better understanding of microorganisms. Microorganisms already familiar in use have been genetically modified to produce biologicals like insulin (Fryklund et al., 1992). New prospects are seen in the environmental releases, including the use of living GMMO in food.

Along with the technical developments the safety aspect of "genetic engineering" has been the topic of many discussions. Starting with concern regarding the safety of laboratory studies, the debate went on to include the safety aspects of industrial use and the intended release of GMMO in the environment.

In Denmark the debate resulted in national legislation, The Gene Technology Act of 1986, to ensure environment and health. At the same time the term GILSP (Good Industrial Large Scale Production) was introduced by the OECD report, "Recombinant DNA Safety Considerations". Later principles regarding the contained use of GMMO presented in the EEC directive 90/219 and environmental release 90/220 have been implemented.

There are some basic considerations to be made, when dealing with the GMMO in relation to human health (Table 1.)

Table 1.

-Identification and detection of the GMMO
-The ability of the GMMO to survive and colonize humans
-The ability of the GMMO to exchange genetic material
-Direct effect of the GMMO on the human host
-Exposure of humans
-Indirect effect of the GMMO on the human host

The general idea of risk assessment includes the exposure of humans to the test substance, the GMMO. A likely exposure route would be by the oral route reaching the gastrointestinal system. The balanced stable microbiota of the mammalian gastrointestine provides "resistance" (Van der Waij et al., 1971) or a "barrier" (Ducluzeau et al., 1970) against colonisation by "new" microorganisms to which the host is exposed. This is to be considered as the main function of the microbiota. The microbiota is only of minor nutritional significance to the host (Coates 1987). At the Institute of Toxicology

different models of the mammalian intestine have been established, exhibiting different degrees of colonisation resistance (CR), ranging from germ-free to conventional rats.

As pointed out in table 1, the ability of the microorganism to survive and the ability of gene transfer in the environment are important parameters in risk assessment. Limitations in survival and gene transfer are the key elements of biological containment. Molecular constructions have been made to improve the detection and the biological containment of GMMO.

The use of *Escherichia coli* and *Lactococcus lactis* as test microorganisms in different gastrointestinal models have enabled the study of detection, survival, colonisation and gene transfer.

The overall objective of the studies presented in this thesis has been to combine biotechnology and classical microbiology with advanced animal science to elucidate the fate and effect of GMMO. The purpose of gaining important knowledge and experience from the testing in animals has been to use this information in the practical implementation of risk assessment.

The aims of the investigations were:

- To study a biological containment system designed for reduced survival of GMMO in the mammalian gastrointestinal tract (Paper I and II).

- To study a biological containment system designed for reduced transfer of genetic material from GMMO in the mammalian gastrointestinal tract (Paper III).

- To study the transfer of genetic material in the mammalian gastrointestinal tract (Paper IV and V).

- To study the use of markers for the detection of microorganisms in the mammalian gastrointestinal tract (Paper V).

- To compare the use of germ-free rats with antibiotic treated conventional rats and/or conventional rats for the study of GMMO (Paper V and Appendix A).

2. RISK ASSESSMENT

Risk assessment of chemicals includes three basic elements:

- Hazard identification
- Risk estimation
- Risk evaluation

Hazard identification is the first step to be taken, and involves toxicological or epidemiological studies to point out the particular hazard. By analysing data from toxicological and epidemiological studies and relating these to human exposure a risk estimate can be made, that is the probability of an event is assessed. This step is followed by risk evaluation taking into consideration current legislation/regulatory options. Finally risk evaluation is followed by risk management (Krewski & Birkwood 1987).

However risk assessment of microorganisms is inherently different from risk assessment of chemicals. This lead to the basic question whether existing models developed for the risk assessment of chemicals are suitable for risk assessment of microorganisms (Mossel and Struijk, 1993, Jacobsen et al., 1994) including the risk assessment of genetically modified microorganisms (Franklin & Previsich 1988).

Classical toxicology as known from the testing of chemicals (OECD guidelines) includes among others studies of:

- Acute toxicity
- Chronic toxicity
- Carcinogenic potential
- Effect on reproduction
- Teratogenicity
- Immunological effects

Considering risk assessment of microorganisms at least two very important additions to the presented list should be made:

- Potential infectivity/pathogenicity
- Ability to persist within the exposed individual

A survey of test methods for the health assessment of microbial plant protection products has been made. The survey lists methods relevant to health assessment, including the USEPA guidelines for testing microbial pest control agents. The survey also includes considerations regarding administration routes, colonisation, toxin production and the use of tier testing in the toxicological testing of microorganisms used as plant protection product (Jacobsen et al., 1994)

Recommendations for the risk assessment of GMMO have been the subject of several working groups. Starting with the risk assessment of recombinant DNA experimentation with *Escherichia coli* resulting in the establishment of specific testing protocols (Gorbach 1978) the work continued internationally. In 1986 the OECD report: Recombinant DNA safety consideration was presented, introducing the concept of GILSP (Good Industrial Large Scale Production). In this report several recommendations/criteria regarding the host, the vector and the recombinant DNA modified microorganism were made (Recombinant DNA Safety considerations, appendix F, OECD, 1986, p. 52).

A Danish working group of scientist was established to point out scientific areas to be elucidated, to make a competent evaluation of the host microorganism in relation to the concept of GILSP (Christiansen et al., 1991a). The work resulted in a risk assessment model, which was used on several well-known industrial microorganisms (Christiansen et al., 1991b). It is important to notice, that the GILSP status was only applicable to the host. The genetically modified microorganism should according to report be evaluated "case by case", as the model did not take the insertion of foreign DNA into consideration.

The concept of case by case risk assessment of GMMO used in environmental releases is generally accepted. In a workshop summery, "the recommendations for a scientific based approach to safety assurance for environmental introductions of genetically engineered microorganism" the OECD definition of a case by case approach was presented with a discussion on the terminology of risk assessment, pointing out that the term "risk assessment" should not be used, unless a plausible risk is identified (Colwell et al., 1988). As the term "risk assessment" is generally used in relation to environmental releases it will also be used in this thesis.

Human health effects research in relation to the deliberate releases of GMMO into the environment as presented in the EPA biotechnology risk assessment research program (Levin et al., 1987) focus on methods to estimate health effects and guidelines for risk assessment. In Denmark the same basic need for more research regarding risk assessment was recognized.

Looking at the different approaches to risk assessment in relation to human health, there seems to be some common keypoints reflecting the nature of microorganisms. Important parameters in risk assessment are the ability of the GMMO to survive and colonize a mammalian host, the ability to transfer genetic material to other organisms and it's direct or indirect effect on the mammalian host.

To study the fate and effect of a GMMO using laboratory animals three elements important to the testing have to be taken in consideration:

-The exposure route
-The test microorganism (GMMO)

-The animal model

If the GMMO is environmentally stable, there will be a risk for human exposure (Levy 1985). Several factors affect the environmental stability, Table 2. Major routes of exposure are: Exposure due to inhalation of the GMMO, exposure by oral intake and exposure of the skin, eyes etc.

Table 2. Factors affecting human exposure and health.

Factors related to the microorganism:

- survival and growth in different ecosystems (colonisation)
- pathogenic traits (adherence, toxin production)
- gene transfer

Factors related to the environment:

- physical factors (temp. pH, O_2 , humidity, redox potential, UV light)
- microbial interactions (competition, predators)

A very important route of exposure will be by the oral route. GMMO may be a constituent of food. Microorganism used as starter cultures may be improved by genetic modification. GMMO designed to have special competitive advantages may be used in biopreservation. GMMO designed to produce specific flavours may be used as food additives. Finally the GMMO from the environmental releases may find their way to the gastrointestinal tract as pollutants.

Deciding studying a particular exposure route, the next step will be to look at the test microorganism. The test microorganisms presented in this thesis represent two different types of GMMO or test microorganisms. *Lactococcus lactis,* used as a starter culture for dairy products, represents the type of GMMO that is foreign to the mammalian host. *E. coli* is a natural habitant of the mammalian intestine. As one of the first well characterised microorganism, it was and still is an important tool used in genetic engineering. In addition it is used for the large scale production of human growth hormone (Dalbøge et al., 1988).

In many studies relevant to risk assessment *E. coli* K12 laboratory strains have been used. *E. coli* K12 was originally isolated from a human. In relation to host specificity (Curtiss 1976), humans should be the obvious choice for testing the recombinant *E. coli* K12. Several studies of *E. coli* K12 include humans (Anderson 1975, Smith 1975, Levy et al., 1980). In other cases of testing GMMO there may be no human data available and studies involving humans are normally not allowed. Instead the use of laboratory animals as in the toxicological evaluation of chemicals may be taken in consideration.

There are three main types of animal models. Type A: a living organism in which deviation/change from the normal physical condition has been induced. Type B: a living organism with a heritable or naturally acquired pathological condition, and finally a proposed type C, transgenic animals, with altered genomes (Meyer 1993).

According to an international working group at a workshop on risk evaluation of genetically modified microorganisms in relation to human health a number of different *in vivo* models and *in vitro* models are available to evaluate the fate and effect of GMMO in human intestinal tract (Benbadis et al., 1995). The next chapter will focus on different type A *in vivo* models of the mammalian intestine and briefly introduce the principles of *in vitro* models.

3. THE MAMMALIAN GASTROINTESTINAL TRACT

The human intestine is a complex ecosystem composed of host cells and their products, food, bacteria, protozoa, fungi and viruses. The gastrointestinal tract has a very high species diversity. The gastrointestinal tract is believed to contain a total of 10^{14} microbes (Luckey 1972) and more than 400 different species can be isolated from the faeces (Moore & Holdemann 1974).

Studies of the mammalian gastrointestinal tract show differences in the normal microbiota of different animal species, not surprisingly reflecting differences in anatomy and physiology (Table 3). In addition some bacteria may exhibit specificity for a particular animal species. However, some major generalizations can be made regarding the mammalian gastrointestinal ecosystem. The native inhabitants, also described as the indigenous, autochthonous microbiota, reside in specific niches in particular habitats of the gastrointestinal tract. Different habitats can be distinguished in the lumen, the mucus, the epithelia or the crypts of Lieberkühn (Savage 1983, Lee 1985).

Each habitat in the adult animal is colonised by climax communities, where the microbial strains may be differentiated into autochthonous or allochthonous types (Savage 1977). This stable climax community is a result of a succession (Savage 1987). The foetus in utero is sterile but already during and after birth, the individual acquires a gastrointestinal microbiota characteristic of its species influenced by the mother (Bennet & Nord, 1989, Tannock et al., 1990, Tannock 1994).

Regulatory mechanisms affect the succession and the climax community. Allogenic factors are regulatory mechanisms exerted by the host, food and environment. Autogenic factors are regulatory mechanisms exerted by the microorganisms (Table 4). Examples of autogenic factors are microbial produced toxins, bacteriocins or the capacity to associate with epithial cells (Savage 1987).

The mammalian gastrointestinal tract can be divided into several distinct areas. Humans are monogastric omnivores with no indigenous microbiota of the stomach due to low gastric pH in contrast to rodents (and pigs) with a microbiota of lactobacilli and streptococci. The small intestine is a long tube in which the mucosa is folded and supplied with finger like villi, by that extending the surface area. The epithelial cells are involved in digestion and absorption functions (Drasar & Barrow 1985, Savage 1989). Between the small and large intestine the tube is supplied with a blind pouch, the caecum. The size and function of the caecum vary depending on the animal species. In man the caecum is very small and constitutes the appendix, but in the rat, the caecum is large and houses a divers microbiota. Caecum and

colon do not contain villi. Instead the colonic mucosa may fold into structures called rugae.

Table 3: Microbiota of different animals, including man (log_{10}cfu/g intestinal contents).

	Stomach (anterior)			Stomach (posterior)		
	rat	man	pig	rat	man	pig
E. coli	3.3		5.3	2.3	N	3.0
Clostridia	N		2.4	N	N	N
Streptococci	5.3		6.0	4.7	N	4.4
Lactobacilli	7.8		8.6	7.2	1.0	7.0
Yeast	6.2		4.3	6.0	1.0	4.3
Bacteroides	N		N	N	N	N
			Small intestine			
		pH=1			pH=3	
	rat	man	pig	rat	man	pig
E. coli	2.2	3.4	2.7	2.7	N	3.7
Clostridia	N	N	N	N	N	N
Streptococci	4.4	N	4.2	4.4	N	4.5
Lactobacilli	6.7	2.0	6.5	7.0	2.0	7.0
Yeast	6.0	1.0	3.9	5.9	1.0	3.9
Bacteroides	N	N	N	N	N	N
		pH=5			pH=7	
	rat	man	pig	rat	man	pig
E. coli	2.9	N	4.5	4.2	5.6	5.3
Clostridia	N	N	1.7	N	N	3.0
Streptococci	5.0	N	6.0	4.4	N	6.5
Lactobacilli	7.0	3.5	7.6	7.2	4.2	8.0
Yeast	6.2	1.0	4.4	6.0	1.0	4.0
Bacteroides	N	N	N	N	5.2	N
	Caecum			Faeces		
	rat	man	pig	rat	man	pig
E. coli	5.9	6.5	6.5	6.0	7.6	6.8
Clostridia	3.0	2.0	N	N	4.0	N
Streptococci	6.4	2.6	7.0	6.2	7.0	7.2
Lactobacilli	7.7	6.4	8.6	8.0	6.4	8.9
Yeast	6.7	2.0	4.0	6.5	1.0	4.2
Bacteroides	8.7	7.9	7.4	8.9	10.0	7.6

N= none detectable

Based on Smith 1965 and Drasar & Barrow 1985

Flowrate, pH and oxygen tension (Eh) are important factors, which influences the site of microbial colonisation. The small intestine contains transient microorganisms and supports the microbiotas of the facultative anaerobic streptococci, staphylococci and lactobacilli. In the distal ileum gram negative bacteria appear. Coliforms are present, and anaerobic bacteria such as Bacteroides, Bifidobacterium, Fusobacterium and Clostridium are found in high concentrations. High bacterial concentrations are characteristic of the colon, in which Bacteroides, Bifidobacterium and Eubacterium

dominate. Anaerobic gram-positive cocci, Clostridia, enterococci and species belong to Enterobacteriacea are common (Drasar & Hill, 1974, Finegold et al., 1983, Drasar & Barrow 1985).

Despite the obvious differences when comparing man and rat (Smith 1965, Rowland et al., 1986, Drasar 1988, Jensen 1992), human microbial populations interact among themselves and with their host in ways similar if not identical to many interactions of the gastrointestinal microbiota of non-human mammals (Savage 1985, Midtvedt 1986, Savage 1989).

Table 4 : Factors influencing colonisation of the gastrointestinal tract.

Allogenic factors	Autogenic factors
Host diet	Lactic acid
Host enzyme activity	Volatile fatty acids
Host immune response	Hydrogen Sulfide
Mucus production and epithelial growth	Nutritional competition
Host temperature	Competitive association with surfaces (Nonspecialised and specialised adherence)
Peristalsis	Bacteriocins
pH	
Bile acids	

Based on Savage 1977, Hentges 1983, Guerina & Neutra 1984 and Savage 1987.

4. MODELS OF THE MAMMALIAN GASTROINTESTINAL TRACT

In ecological terms, species diversity, is a community parameter, that relates to the stability of that community. Communities with too much or too little diversity would according to Atlas be subject to continuous or catastrophic change (Atlas 1983). A high diversity index as found in the mature animal ensures the higher capacity to counteract on environmental changes. This would include the constant challenge of the gastrointestinal ecosystem with environmentally introduced microbes.

The stable climax community of the mammalian gastrointestine helps the animal and human to resist infections. These phenomena have been described by various authors and given the names bacterial antagonism (Freter 1956), bacterial interference (Dubos 1963), barrier effect (Ducluzeau et al., 1970), colonisation resistance (Van der Waaij et al., 1971) and competitive exclusion (Lloyd et al., 1977).

In this thesis the term colonisation resistance (CR) will be used. The original definition of CR gives the numbers of bacteria (log values) by oral intake that results in colonisation for more than two weeks of 50% of the animals dosed. The term originally proposed in 1971 by Van der Waaij is no longer regarded as an quantitative term. Instead the term rather reflects the stability of the ecosystem in relation to colonisation by exogenous microorganism (Freter 1984a, Lee 1985).

Numerous studies have tried to elucidate the exact mechanism(s) of CR. From Van der Waaij originates the hypothesis, that CR was related to the presence of the anaerobic microbiota (Van der Waaij et al., 1971). Later the influence of the immune system on CR was added to the factors affecting the microbiota and therefore the CR (Van der Waaij 1988, Van der Waaij 1989). The gastrointestinal tract is equiped with an extensive immune apparatus. Antigen uptake through the epithelia may result in the production of IgA class antibodies or the suppression of systemic immunological responses to ingested antigens ("oral tolerance").

Investigators have found that besides the anaerobic bacteria, aerobic and facultative anaerobic bacteria may also have a role in CR (Wells et al., 1988). Early studies suggested that the competition for nutrients may be one of several mechanisms contributing to observed "bacterial antagonism". Filtered medium from *E.coli* cultures was shown to inhibit the growth of *Shigella flexneri* under reducing conditions (Oxidation-reduction potentials in the range of -100- -150 mV). This inhibition of Shigella by filtered *E. coli* medium could be reversed by aeration or by the addition of glucose (Freter 1962). Other studies have focused on the competition for attachments sites (Freter 1983, Freter et al., 1983a)) and the bacterial production of toxic or inhibitory metabolites like volatile fatty acids (Que et

al., 1986).

In conclusion - multiple factors affect the colonisation and diversity of the climax community - therefore multiple factors will contribute to the CR. In addition to the factors given in table 4 the longevity of the exogenous (invading) bacteria, that enters the gastrointestine, is important. Lag phases may last for some time resulting in the washout of bacteria before they can multiply. Finally some pathogens are capable of overcoming CR resulting in ecosystem disturbance and disease.

Mathematical models, *in vitro* models and *in vivo* models have been developed and used for the study of the basic principles for bioregulation and microbial interactions in the gastrointestinal tract. The use of *in vitro* models of the gastrointestine varies from simple batch models, based on faecal suspension (Boriello et al., 1988) or mucus preparations (Wadolkowski et al., 1988) and small anaerobic continuous cultures (Freter et al., 1983c) to complex two stage chemostats (Veilleux & Rowland 1981, Nielsen 1991, Schlundt et al., 1992). An extremely sophisticated *in vitro* models is the artificial gastrointestine developed by TNO (Veenstra et al., 1993).

The *in vivo* models of the mammalian gastrointestinal microbiota may be divided into three major groups, Table 5.

Table 5: *In vivo* models of the mammalian gastrointestinal microbiota.

Group	CR	Terminology	Microbial status
1	High	Conventional (CV)	Stable unknown microbiota
2	Altered	Antibiotic treated CV	Disturbed unknown microbiota
3	None or low	Gnotobiotic	Wellknown microbiota

The groups presented cover in principle a variety of species including the rat.

The conventional rat has a stable diverse climax microbiota and thus represents an ecosystem with a high CR, as pointed out in the beginning of this chapter. The term conventional is used in a broad sense in this thesis. When obtained from the breeding facilities of Moellegaard (Moellegaard Breeding Centre Ltd. DK-4623 Ll. Skensved, Denmark) they are characterised as SPF, that is, they are free from specific pathogens. A list of terminology is given in Appendix C.

Many antimicrobial agents are known to disrupt the ecological balance of the intestinal microbiota. This may result in altered CR and therefore

colonisation by exogenous microorganisms or outgrowth of certain indigenous microorganisms. This knowledge from studies in animal and man has been used in the development of animal models to study the colonisation capacity of specific test bacteria.

The effect of antimicrobial agents is not only seen as changes in microbial composition and diversity. The influence of the microbial agents is also seen as changes in microflora-associated characters (MAC) in animal and man (Midtvedt 1985, Steinbakk et al., 1992).

The third group - the gnotobiotic animals - reflects the lowest microbial diversity and level of CR. The group of gnotobiotic animals, defined as animals with a known microbiota, include germ-free animals. The definition of a germ-free animal is: An animal free from any other detectable form of life (Gustafsson 1984). This microbial status is difficult to achieve and the maintenance of the microbial status requires the use of a sterile environment (Gustafsson 1948, Jacobsen 1992).

When microorganisms are absent, as in the germ-free animals, the recording of a microflora-associated characteristic (MAC) can be defined as a germ-free animal characteristic (GAC) (Midtvedt 1985). Table 6 summarises some intestinal structures and functions influenced by the microbiota.

Germ-free and gnotobiotic rodents have been used in broad range of experiments ranging from elucidating the influence of the gastrointestinal microbiota on toxin production in *Clostridium difficile* (Corthier et al., 1986) to elucidating the physiological importance of the colonic microbiota on the host (Gustafsson 1982).

An important characteristic of the germ-free animal is, that almost any solitary introduced microorganism, capable of growing in the gastrointestinal environment, will be able to colonise the intestine due to the optimal conditions for colonisation provided by the germ-free animal. Very high bacterial concentrations in faeces are reached (10^9-10^{10} cfu/g) within 24 hours after dosing (Ducluzeau 1984, Jacobsen 1992).

Several of the GAC have been investigated and compared with corresponding MAC in material from germ-free and conventional rats and from healthy children and adults. These studies reveals similarities between germ-free rats and newborn babies in some functional aspects of the intestinal microbiota, like the missing degradation of mucin and the missing excretion of coprostanol and urobilinogen (Norin 1985). The germ-free rat may be regarded as a highly specialised animal model, but it still has relevance to its human counterpart. In that respect it may be regarded as a "worst case model", simulating a situation of no protective microbiota.

Table 6: Some intestinal structures and functions influenced by the microbiota.

	MAC	GAC
Caecum size	Normal	Enlarged
Passage time	Normal	Increased
Intestinal wall	Thick lamina propria Irregular villi High cell turnover	Thin lamina propria Regular villi Low cell turnover
Immunoglobin	Normal contents	Low contents
Lymphnodes	Normal	Less developed
Mucin	Absent in faeces	High amount
Volatile fatty acids	Several acids High amounts	Few acids Small amounts
Bile acid metabolism	Deconjugation Hydroxylation	No deconjugation No dehydroxylation
Bilirubin metabolism	Deconjugation Urobilinogen	No deconjugation No urobilinogen
Cholesterol	Mainly coprostanol	Cholesterol
Intestinal gases	H_2, CH_4, Co_2	No H_2 or CH_4. Reduced CO_2
Tryptic activity in faeces	Little or no activity	High activity

MAC= Microflora associated characteristics
GAC= Germ-free animal characteristics

After Midtvedt 1986 and Coates & Gustafsson 1984.

5. BIOLOGICAL CONTAINMENT

Biological containment is an important concept in the risk assessment of GMMO. Factors affecting biological containment are the ability of the GMMO to survive and disseminate in the environment and the ability to transfer genetic material to other organisms in the environment. The degree of biological containment depends on an evaluation of both the host and the vector (Jacobsen 1994). To show a high level of biological containment, the modified microorganism must be very limited in survival and restricted in its ability to transfer genes.

The strategy of biological containment will be different depending on the use of the GMMO. The containment principle used on microorganisms in industrial fermentations may not be the same for GMMO used in the environment. Biological containment using a disabled host has its limitations. Disabled bacteria used in industry are not expected to survive in the environment, however some may survive. A contrast to physically contained use is the deliberate environmental release, where it is essential that the GMMO survive and perform. Rather the problem is to programme a microorganism to express the desired phenotype at the correct level and at the right time. This may even be required in environments that are beyond direct human control. Deliberate releases comprises a lot of situations ranging from bioremediation, live vaccines to the intake of GMMO as food.

This problem of desired control of survival, colonisation and gene transfer can be solved be the introduction of controlled suicide functions into a competitive wildtype. The suicide functions do not interfere with the normal growth and functions of the bacterium, and it is only upon specific signals that the suicide function is activated.

Several systems have been developed. Some systems are capable of limiting the survival of the bacteria, others restrict specifically the transfer of plasmids. Finally constructions have recently been made to limit survival to a specific environment and at the same time eliminate the possibility of gene transfer. A biological containment system resulting in bacterial suicide consists of to elements. The genes coding for the killing function and the regulatory genes that control the expression of the killing genes.

The killing genes belong to the *gef* gene family. This family includes three genes *hok*, *gef* and *rel*F used in different suicide systems (Molin et al., 1987, Knudsen & Karlström 1991, Jensen et al., 1993, Klemm et al., 1995). The genes have homologous sequences and were all isolated from *E. coli*. The isolated killing gene *hok* (Host killing) was found on the plasmid R1 (Gerdes et al., 1986) and showed familiarity to the *relf* gene located the *E. coli* chromosome (Bech et al., 1985). Finally the *gef* (gene expression fatal) gene was isolated and characterized. The *gef*-homologous sequences were found conserved in other gram-negative species indicating an important

physiological role (Poulsen et al., 1989).

Expression of the *hok* and *gef* genes in *E. coli* results in the killing of cells and the appearance of ghost cells (Gerdes et al., 1986, Poulsen et al., 1989). The genes code for very small lethal proteins, probably targeted for the cell membrane. The *hok* gene has shown to be lethal to several other bacteria including *Bacillus subtilis* (Molin et al., 1987). Studies using the *gef* gene have indicated that problems of gene expression, rather than sensitivity of the cellmembrane, result in survival of *Bacillus thuringiensis* (Molin et al., 1993).

Activation of genes coding for nucleases is a very promising possibility for biological containment. Activation of a nuclease coding gene will not only results in the killing of the cell, but also destroy the DNA, thereby eliminating possible gene transfer. Such a system based on the activation of *nuc* gene from *Serratia marcescence* has been constructed and used in *E. coli* (Ahrenholtz et al., 1994). Others have used the *Bacillus subtilis sac*B gene to construct a conditional suicide system for *E. coli* released into soil. The *sac*B gene results in sucrose sensitivity when expressed in gram-negative cells. The gene, coding for levansucrase, is inducible in the presence of sucrose resulting in accumulation of levan resulting in cell lysis (Recorbet et al., 1993).

The main problem of the suicide systems is the proper expression of the killing function when the bacteria are used in the environment. Several different attempts have been made to solve this problem reflecting the intended use.

Chemical induction of the killing by an inducible promoter has been made. Examples of this are the use of the tryptophan promoter, where absence of tryptophan in culture medium, leads to the activating the *hok* gene (Molin et al., 1987), the use of lac promoters, where the *rel*F gene is activated upon induction by ITPG (isopropyl-beta-D-thiogalactopyranoside) resulting in suicide (Knudsen & Karlström, 1991) or the *sac*R-B cassette inducible by sucrose (Recorbet et al., 1993).

Physical control as in the form of induction by a rise in temperature has also been used. The lambda p_L promoter has been used for thermoinduction of a nuclease gene derived from *Serratia marcescence*. This promoter will be repressed at 28°C in an *E. coli*, which carries the thermosensitive lambda cI857 repressor. A temporary rise in the incubation temperature from 28°C to 42°C resulted in the activation of the nuclease gene causing intracellular DNA degradation leading to the death of *E. coli* cells. The elimination of cells was not complete, but most of the *E. coli* were responsive to a second induction leading to further reduction in number of cells (Ahrenholtz et al., 1994). Such systems may be suitable for the use in laboratories or in industry at the end of a fermentation.

In natural environments the situation is far more complicated (De Lorenzo 1994). Several researchers have worked on expression system involving the

response to external signals present in the environment. Potential transfer of recombinant DNA (rDNA) to the native microorganisms is reduced, when the rDNA is located chromosomally. Therefore many suicide system are constructed as cassettes ready to be inserted chromosomally.

In a substrate dependent biological containment system for *Pseudomonas putida*, the *gef* gene is coupled to a *lac*-promoter. This encoded killing function has been integrated on the chromosome. A plasmid designed to control the killing function acts on an environmental signal. This is achieved by a construction coding for the *xyl*S2 and the *lac* repressor. XylS2 is known to be a positive regulator of the Pm, the meta-cleavage pathway operon for the catabolism of benzoate and certain alkylbenzoates to Krebs cycle intermediates. The LacI protein is made in the presence of a XylS effector like m-methylbenzoate, and thus prevents the expression of the killing system. If the substrate is missing, then the repressor is no longer synthesized and the killing function activated (Jensen et al., 1993). This suicide system may be relevant to bioremediation.

A suicide system based on the stochastic activation of the *hok* gene (Molin et al., 1987) or the *gef* gene (Klemm et al., 1995) has been envisaged for the containment of live genetically modified vaccines (Eisenstein et al., 1992) as well as for industrial productions using *E. coli* (Klemm et al., 1995). These constructions are based the invertible switch promoter, *fim*A, isolated from the regulatory sequences involved in fimbria expression. Fusion of this promoter with other regulatory genes from the *fim* operon and a killing gene, ensures the random activation of the killing function and thus lead to a reduction of the bacterial population.

Complete elimination of microorganisms by suicide systems has been extensively discussed. Apparently there is no system so far that guarantee total cell death. All the systems tested so far demonstrate the presence of survivors. Several attempts have been made to reduce this survival. Assuming that survival is due to mutations in the killing gene, two copies of the killing gene instead of one reduces the number of survivors (Knudsen & Karlström 1991, Jensen et al., 1993).

The studies presented in this thesis elucidate different aspects of biological containment. The studies elucidate the effect of killing genes on survival, colonisation (papers I, II) and transfer (III). The above described systems based on the stochastic activation of the killing genes *hok* and *gef* are expected to result in limited survival and colonisation of the contained GMMO. The *in vivo* testing of such reduced ability to survive and colonise the gastrointestinal tract of mammals is the subject of the next chapter.

6. STUDY OF SURVIVAL AND COLONISATION OF THE GASTROINTESTINAL TRACT

A prerequisite of pathogenicity in general is the ability of the pathogen to overcome the host defense, to colonize a particular niche and to express virulence traits. To be epidemic the GMMO must also be able to survive and be transmitted to other individuals (Curtiss 1978).

Studies of *E. coli* in animals and in man have shown that colonisation by exogenous *E. coli* is difficult under normal conditions due to the protective indigenous microbiota. However it has also been stated, that virtually any strain of *E. coli* under the appropriate conditions has the intrinsic ability to colonize the large intestine of animal and man. This relative ability to colonize may be dependent upon a number of microbial and host parameters (Laux et al., 1982).

Studies of competition between congenic *E. coli* K12 strains in gnotobiotic mice have clearly shown that limited genetic alteration can enhance or reduce the relative colonisation capacity. As an example *E. coli* strains differing in chromosomal located genes coding for antibiotic resistance showed different colonisation ability. This observation may be related to changes in the membrane proteins (Onderdonk et al., 1981).

Other studies of expression of plasmids carrying antibiotic resistance genes, have shown that expression of resistance to tetracycline can affect the fitness of the plasmid containing bacteria in an antibiotic-free medium (Lee & Edlin 1985). These studies were performed in the laboratory. In nature it is much more difficult to assess the influence of plasmids on the colonisation of the intestine. Several studies have shown, that in general, plasmids do confer an ecological disadvantage to the host bacterium (Duval-Iflah & Chappuis 1984). However this may be to general a statement to be made. What may be a disadvantage in one situation, may be an advantage in another situation, thereby reflecting flexibility and explaining the abundance of plasmids in nature constituting an important gene pool.

An interesting observation was made in relation to the studies of the effect of plasmids on colonisation. When isogenic strains of *E. coli* were competing in the intestine of gnotobiotic mice, the outcome depended on the order in which the strains were introduced. The strains were given by with an interval of seven days. This intra species interaction gave the first introduced strain an advantage (Duval-Iflah et al., 1981).

Systems of high degree of biological containment were designed in the 1970s for laboratory use. *E. coli* K12, originally isolated from a patient at Stanford Hospital in 1922, was in 1978 the only strain to be used for inserting foreign genetic material according to the NIH guidelines (Curtiss 1978).

Several *E. coli* K12 strains were studied for their ability to colonise humans and gnotobiotic animals (Anderson 1975, Levy et al., 1980). In general *E. coli* K12 are very poor colonisers. Due to mutations in widely spaced gene clusters on the chromosome, the *E. coli* K12 strains have lost their ability to express O- and K-antigens, antigens associated with virulence and ability to colonise. The serotype of a K12 strain should be written O-: K-: H48 (Ørskov 1978). Other mutations have lead to special growth requirements and increased sensitivity to UV-light. To use an *E. coli* K-12 strain for testing a plasmid carrying a biological containment system based on the activation of suicide genes would not be an optimal choice. Instead a vigorous wildtype isolated from the environment would provide the maximal challenge to the containment system.

Wildtype *E. coli* was isolated from various animal populations at the Institute of Toxicology. To qualify as test microorganism the isolate had to fulfil several requirements. The *E. coli* should be non - pathogenic to man and animal. It should be able to be transformed using pBR322. The *E. coli* should preferably have to be isolated from a rat, to ensure intrinsic colonisation capacity, because the rat was chosen as the laboratory animal model. Finally the isolate should not carry any antibiotic resistance genes typically used in genetic engineering. Wildtype isolate *E. coli* BJ4 from a Wistar rat met also these criteria (Jacobsen 1992). The microorganism has been the basis for the study of biological containment systems using germ-free rats described in the papers I and II.

In vitro studies using laboratory strain of *E. coli* had showed an effect of the suicide system based on the activation of the *hok* gene (Molin et al., 1987). The important question was: Would it also work in real life - that is - would it work in a wildtype *E. coli* in the natural habitat, the gastrointestinal tract. The answer is presented in paper I. The germ-free rat was chosen for the study due to several experimental advantages. The gastrointestinal tract is a complex biological environment compared to the test tube. The lack of a diverse microbiota gives the advantage of easy detection and allows the study of the test microorganism alone or in competition.

E. coli BJ16 contains a plasmid pPKL100 but is otherwise identical to *E. coli* BJ4. *E. coli* BJ16 were given to germ-free rats. The plasmid pPKL100 carries the hok gene under control of the *fim*A promoter. This will result in a random activation of the *hok* gene. Resistance to the antibiotic ampicillin was used as marker for this construction (Molin et al., 1988). The monoassociation with *E. coli* BJ16 did not result in the elimination of *E. coli* BJ16 from the gastrointestinal tract. Instead a plasmid-free and a plasmid-containing population were formed and coexisted in the gastrointestinal tract.

In contrast when *E. coli* BJ16 was given to gnotobiotic rats initially associated with *E. coli* BJ4, the BJ16 was reduced. This design was intended to illustrate a "real life" situation, where the GMMO will compete with the resident microbiota, exemplified by its original counterpart. The *E. coli* BJ16(*hok*+) was eliminated at a faster rate than *E. coli* BJ17 in a similar experimental situation. *E. coli* BJ17 is identical to BJ4, but carrying a

plasmid pMG33 without the hok gene. This construction is marked by resistance to the antibiotic tetracyclin. Expressed as T_{90}, the time used for a 90% reduction of the bacterial concentration, the mean elimination for *E. coli* BJ16(*hok*+) was 2.8 days compared to 5.3 days of *E. coli* BJ17(*hok*-). This indicated an effect of the *hok* gene in a situation, where the contained microorganism was competing with its natural counterpart.

The study of the *hok* based biological containment study raised a number of questions. The experimental design did not allow a direct estimation of loss of plasmid. The use of different antibiotic resistance markers may also have influenced the growth of *E. coli*. The expression of the tetracycline resistance gene could have an adverse effect on the reproductive fitness of plasmidcontaining *E. coli* (Lee & Edlin 1985). At the same time Molin and co-workers at the Technical University continued optimizing the biological containment systems.

We decided to continue the investigations using constructions based on the activation of the *gef* gene. The activation of the *gef* gene was accomplished by the use of an invertible promoter sequence of the *fim*A gene. This sequence acts as a switch with an "off" or "on" configuration. The construction also included the regulatory genes *fim*B and *fim*E. The *fim*B gene product mediates an "on" configuration and the *fim*E gene product mediates an "off" configuration of the *fim*A promoter (Klemm et al., 1995). Similar to the investigations of the effect of the *hok* gene, the experimental design was based on a measurement of the effect of the suicide gene expression on the decimation time of an exogenous strain in the gastrointestine of the gnotobiotic rat (Paper II). The results of the study were in accordance with the results of previously obtained with the *hok*-gene (Table 7). In addition, using bacillus spores as a marker for intestinal transit time, we were able to demonstrate, that the *gef* gene contained in *E. coli* in the gastrointestine causes bacterial death in contrast to multiplication of *E. coli* without the suicide gene.

Table 7. The effect of suicide genes *hok* and *gef* on the decimation time T_{90} of *E. coli*.

n	Plasmid	T_{90} (days)	SEM	P(>t)
7	gef+	2.5	0.5	0.0017**
7	gef-	6.6	0.9	
10	hok+	2.8	0.3	0.0048**
5	hok-	5.3	0.8	

T_{90} = the time used for 90 per cent reduction in bacterial concentration.
SEM = standard error of the mean.
** Statistically significant difference using a *t* test.
P(>t) = probability for a higher t value under the hypothesis: T_{90} values are equal.
n = number of rats

The experimental design of the studies of the *hok* and *gef* based containment systems is important for the interpretation of results. The design used in

paper I and II simulates a situation, where the "normal" *E. coli* population are exposed to the GMMO. In appendix A-III the results of another design are presented. Again germ-free rats are exposed to the same *E. coli* strains used in paper II. In contrast to the design in paper II, the wildtype strain E. coli BJ4 and the *E. coli* with the *gef*-gene (BJ19/pSM910) were given at the same time to the germ-free rat. As pointed out earlier by Duval-Iflah et al. the outcome of intra species competition depends on the order of inoculations with a seven day interval (Duval-Iflah et al., 1981). The two strains will have equal opportunities to colonise the gastrointestinal tract. This design may be considered the "worst case" situation, where no protective microbiota preexists. The results of the study are in contrast to the results observed in paper II. The concentrations of *E. coli* with the *gef*-gene (BJ19/pSM910) in faecal samples are not reduced over time, instead the concentrations remain high (approximately 10^9cfu/ g faeces) and stable for at least 27 days. The conclusion of the study is that no effect of the *gef*-gene is observed in a situation, where the *E. coli* has the same competition conditions as the original wildtype *E. coli*. The growth rate exceeds the death rate.

The results can be explained from the following hypothesis: All attachment sites/ residential niches in the gastrointestinal tract are vacant and the nutrients are in surplus in the germ-free rat. All *E. coli* will find space for establishment. A prerequisite will be that the plasmid with the *gef*-gene does not confer a specific disadvantage to the *E. coli* and that the strains have identical physiological capabilities. Monoassociation of the rat with *E. coli* BJ4 will result in an occupation of all residential niches. No attachment sites/ residential niches are left over for secondly administered *E. coli* strains with or without the *gef*-gene. The nutrients may be sparse as they use the same nutrients. The limitations results in the washout of the new arrived *E. coli* BJ19 with or without the *gef*-gene. The growth rates of the BJ19 with or without the *gef*-gene are affected by the situation. The reduced growth rate can not mask an effect of the activated *gef*-gene. The *E. coli* with the *gef*-gene will be washed out faster than *E. coli* without the activated *gef*-gene, as the death rate exceeds the growth rate, thereby adding to elimination. Administered simultaneously the BJ4 and BJ19/pSM910 are able to equally share niches and nutrients. Both strains are established and the high growth rate of strain BJ19/pSM910 masks the death induced by the activation of the *gef*-gene. No wash out of the *E. coli* with the *gef*-gene will be observed.

The overall conclusion of these studies is, that by using gnotobiotic rats we were able to demonstrate an effect *in vivo* of the biological containment system used. The effect was observed in a situation of competition with an existing intra species population. The study with the *hok*-gene based biological containment system in *E. coli* monoassociated in gnotobiotic rats in addition demonstrated the need for this type of studies. The suicide gene containing *E. coli* did not die out. This may have been expected from the results of the earlier made *in vitro* experiments.

7. STUDY OF GENE TRANSFER IN THE GASTROINTESTINAL TRACT USING GERM-FREE RATS

Besides the possible survival and colonisation of the intestine by GMMO, transfer of inserted genes to the host and to the native microorganisms have to be considered. Horizontal gene transfer may be mediated by conjugation, transduction or transformation.

Transformation is the transfer of genes as naked DNA. Eucaryotic cells shed into the intestinal lumen undergo degradation, including a rapid and extensive degradation of DNA. This observation lead to the assumption that uptake of DNA by the mammalian host cells was to be considered very unlikely (Hoskins 1978). This may not be the situation between microorganisms. Natural genetic transformation in the environment has recently been reviewed by Lorenz and Wackernagel. The intestine with its high bacterial concentrations may favour transformation by free DNA (Lorenz & Wackernagel 1994).

Transduction - gene transfer mediated by bacteriophages, may also take place in the mammalian intestine, but the best known/reported mechanism of gene transfer in the intestine is conjugation. Conjugation is the mating between bacteria. It requires cell-to-cell contact and specific genes coding for transfer functions to transfer of DNA from the donor to the recipient.

From studies of the R-plasmid (R-factor) and the dispersal of antibiotic resistance genes it can be concluded that conjugation takes place in the mammalian intestine. This conjugation is between genera of the family of Enterobacteriaceae. The gene transfer is favoured by antibiotic treatment if the used antibiotic will favour bacteria carrying the relevant resistance gene. However transfer of a R-factor to resident *E. coli* has also shown to take place in the human gastrointestinal tract without antibiotic treatment (Smith 1969). Apparently transfer may take place even in the absence of a selective pressure.

Possible plasmid transfer from recombinant *E. coli* K12 strains to resident *E. coli* in the human gastrointestinal tract has been of concern. This concern lead to investigations of the viability of *E. coli* K12 and of the transfer of plasmid from this strain. The study of faecal samples showed that *E. coli* K12 could survive several days. Transfer of plasmids to the resident *E. coli* may have taken place in the human gastrointestinal tract (Anderson 1975).

Several factors are known to affect conjugal plasmid transfer in the intestine. Bacteroides were shown by J.D. Anderson to inhibit R-plasmid transfer *in vitro*. Formalintreated (dead) Bacteroides did not have the same inhibitory effect on transfer. The mechanical effect of inert material was shown to have no major effect on transfer, so the effect on transfer may be restricted to

biologically active Bacteroides (Anderson 1975). This was not so in a later *in vivo* study using gnotobiotic mice. Bacteroides did not inhibit plasmid transfer. The *in vivo* study also made it clear, that even a transient donor, *Serratia liquefaciens,* may donate a R-plasmid to the resident *E. coli* (Duval-Iflah et al., 1980).

The ability of donors and transconjugants to colonise the gastrointestinal tract and to adhere to the epithelia was shown to be important for plasmid transfer *in vivo*. Adhesion to the gastrointestinal wall was by Freter shown to compensate for the reduced fitness of the plasmid carrying *E. coli*. This resulted in a stabilised population of plasmid carrying *E. coli* (Freter et al., 1983a). Freter has worked on the creation of reliable mathematical models of transfer in the gastrointestine. Using a mathematical model on the human data published earlier by E.S. Anderson (Anderson 1975), he concluded, that plasmid transfer did occur in the human gastrointestinal tract. However the resulting transconjugant *E. coli* population was to small to be detected by the used culture methods (Freter et al., 1983b).

The nonconjugative plasmids like pBR322 and pBR325 used with *E. coli* K12 strains have shown not to be transferred (Levy et al., 1980, Levine et al., 1983). However, in a study using a non K-12 *E. coli* strain it was shown that pBR325 may be transferred to other microorganisms by assistance from an incoming conjugative plasmid (triparental mating). This is possible due to the presence of genes coding for proteins used in mobilization (*mob* genes) on the nonconjugative plasmid. Transfer of pBR325 in the presence of a conjugative plasmid has been observed using human volunteers (Levine et al., 1983).

A general statement regarding plasmid transfer has been made by Freter: "Very little or no detectable plasmid transfer occurs in the normal gut populated with an undisturbed microflora. In contrast, when the microflora is absent, as in germ-free or newborn animals, or when it is incomplete or disturbed, as in the very young or in antibiotic-treated individuals, then plasmid transfer can be observed as readily as during *in vitro* matings" (Freter 1984b).

With this statement in mind, we decided to test a biological containment designed for preventing plasmid transfer in the gastrointestinal tract of the germ-free rat. Theoretically the germ-free rat would provide optimal conditions for the *in vivo* transfer of a plasmid. Pilot experiment in germ-free rats using *E. coli* BD3379 as donor and *E. coli* BJ19 as recipient showed it possible to demonstrate a transconjugant *E. coli* strain as a result of gene transfer. The identifications were based on the presence of distinctive markers coding for resistance to antibiotics enabling us to separate the different strains and the conjugative plasmid.

Having developed such a testsystem we were also able to demonstrate the lack of transfer under similar experimental conditions. A biological containment system that prevents plasmid transfer to wildtype *E. coli* was tested. The biological containment system is based on the activation of the

relF killing gene. The suicide functions are present in duplicate on a plasmid to prevent inactivation of the killing function by mutations. The killing function is controlled by the presence of a synthetic promoter, which is efficiently repressed by the lac repressor. The repressor producing gene *lac*Iq1 is present on a second plasmid pR2172. Both plasmids were present in the donor strain.

The presented paper III includes the summation of the results of the investigations using gnotobiotic rats. The suicide functions were constructed in a debilitated laboratory strain (S17.1) of *E. coli* with a very low colonisation potential. Even in the germ-free rat it was a poor coloniser. Repeated oral dosing was necessary to ensure a relatively high concentration of the donor. In contrast, the recipient *E. coli* BJ19, a derivative of *E. coli* BJ4 was an excellent coloniser. To provide the optimal conditions for plasmid transfer rats were first dosed with the donor and later the recipient. No transconjugants were found in the experiment using the contained plasmid.

The use of germ-free rats for the study of microorganisms is not restricted to test microorganisms considered natural inhabitants of the gastrointestine. Gene transfer between different strains of *Lactococcus lactis* have also been demonstrated using gnotobiotic rats (Paper IV).

Lactococcus lactis ssp. *lactis* and *L. lactis* ssp. *lactis* biovar *diacetylactis* has frequently been isolated from plant material. Lactococci are not usually found in faeces. Raw cow milk often contains *Lactococcus lactis* ssp. lactis and *L. lactis* ssp. *lactis* biovar *diacetylactis*. Lactococci are used in the dairy industry as starter cultures for the production of cheese and other dairy products. Daily intake of such products would result in the presence of these microorganisms in the gastrointestine.

Similar to the design in the investigation of the biological containment systems based on the activation *hok* and *gef*, a strain of *Lactococcus lactis* MG1614 was established in the intestine simulating a resident microbiota. The strain MG1614 acts as a recipient. After 29 days a second strain of *Lactococcus lactis* illustrating a transient foodborne microorganism was given to the animals. This strain NCDO712(pAMß1) contains a conjugative plasmid pAMß1 and acts as donor. Due to the observed rapid decline in concentration of the NCDO714 (pAMß1) in faecal samples the donor was re-administered to the rats.

Transconjugants were found in the faecal samples after the initial dosing of the donor. After the re-administration of the donor a stable population of transconjugants were established in a concentration of 10^4 c.f.u./g faeces. This concentration of transconjugants was found throughout the gastrointestine. In contrast the recipient was not uniformly distributed in the gastrointestine. The concentrations of transconjugant and recipient were almost the same in the jejunum, whereas the recipient concentration was 10^4-10^5 higher in caecum, colon and faeces. This finding indicates that the plasmid confers a selective advantage to the recipient in the small intestine.

Another explanation may be that the small intestine is the hot spot for the transfer event. The plasmid confers a selective disadvantage to the transconjugants reducing the number of transconjugants to recipients in the large intestine.

The overall conclusion of these studies is, that it is possible to demonstrate plasmid transfer and lack of transfer between related bacteria in the gastrointestinal tract of mammals using gnotobiotic rats.

An important key, not only to the studies of biological containment systems, but in particular to the studies of gene transfer, has been the use of markers. Traditionally, genes coding for resistance to different antibiotics have been used. The next chapter will focus on the use of markers and methods of detection.

8. STUDY OF ALTERNATIVE MARKERS USING GERM-FREE RATS

Correct detection is fundamental not only to the presented studies, but also in relation to practical application in environmental releases. The general objective is to distinguish specific bacteria from many other bacteria. A prerequisite for most methods is the cultivation of the live bacteria.

The germ-free rat is characterised by the absence of detectable microorganisms (Gustafsson 1984). Cultivation of faecal samples from the germ-free for sterility control illustrates the nonselective principle of cultivation. Optimal conditions for the growth of microorganisms are wanted. In practice this is achieved by cultivation in different media, temperatures and atmospheres (Table 8).

Table 8. Basic cultivation principles for faecal samples in sterility check of germ-free rats.

Media	Temperature	Atmosphere	Incubation time
BHI*	37°C	Semi-anaerobic	3 wks
Thioglycollate	37°C	Anaerobic#	3 wks
Sabouraud	Room temperature	Semi-anaerobic	3 wks

*) Brainheart infusion broth
#) A Wise anaerobic working station (Don Whitley Scientific Ltd.) .

The germ-free status of the rat makes it easy to detect the associated bacteria as compared to the conventional rat. The need for correct identification is however still necessary if one studies more than one bacterial strain at the same time in the rat.

The presented studies all rely on the identification of a specific strain. This was done by a selective principle in the cultivation of faecal or other gastrointestinal samples. Specific media in form of MacConkey agar plates for *E. coli* and GM17 agar plates for *Lactococcus lactis* were used. The addition of different antibiotics to the plates enables differentiation of the bacterial strain according to their genes coding for antibiotic resistance.

Genes coding for resistance to different antibiotics are well-known markers used in genetically modified microorganisms. This type of marker genes may still be very useful under contained conditions in the laboratory. In relation to environmental releases it is very problematic to justify their use.

World wide problems in the treatment of life threatening infections have

focused on the overwhelming emergence of pathogens resistant to treatment with multiple antibiotics. This has resulted not only in the awareness of the spread of genes coding for the resistance, but also on the use of antibiotics including the use of antibiotics as feed additives.

As illustrated in the studies presented (papers III, IV, V) gene transfer in gastrointestinal tract is possible. The studies confirm the observations of several investigators, that genes coding for antibiotic resistance do spread between microorganisms by transfer mechanism (conjugation) in the gastrointestinal tract. To use antibiotic resistance genes in GMMO used for food it not considered acceptable. A report from the Advisory Committee on Novel Foods and Processes UK, July 1994 recommend that such markers are not in use.

Several alternatives to the antibiotic resistance markers have been developed. Several methods for the detection of GMMO have been developed for the tracking of the GMMO in soil, sediments and water. The methods may however also be of relevance to the detection of GMMO in the gastrointestinal tract. The methods can be divided in two major groups: Methods based on phenotypic detection and methods for nucleic acid detection.

Several phenotypic markers besides the antibiotic resistance markers are known. Genes coding for metabolic enzymes have been used as markers. The *lac*ZY genes encoding the ß-galactosidase and the lactose permease are well-known markers. Bacteria containing lacZY can form blue colonies on the colourless media containing X-gal (Drahos et al., 1986). Others have used insertion of the luciferase genes *luc* or *lux*, so that the GMMO can be identified by light emission (Møller et al., 1994). These types of markers have found their use in environmental studies, where GMMO fate and effect have been studied in sediments, soils and seawater.

GMMO used in foods has to be identified in the product during and after the passage of the product through the consumer. A nonselectable marker based on the identification of a specific altered gene sequence has been developed. The identification by such markers is based on molecular techniques, including specific probe hybridization and polymerase chain reaction PCR based methods. A review of nucleic acid based detection systems for genetically modified bacteria have recently been presented (Ludwig et al., 1995).

Hybridization techniques are based on the ability to anneal by specific base pairing of the nucleic acids in the separate but complementary strands. Double stranded DNA strands are formed depending on several factors including temperature and ionic strength. The strands can be separated by denaturing agents. The stability and formation of hybrids will depend on the homology between the nucleic acid sequence of the probe and the target strain. Probes can be artificially made. Such probes, synthetic oligonucleotides, often consist of 15 to 30 nucleotides. Examples of specific probes used to identify a modified gene from its natural counterpart are

PLC1 and PLC2 used in the presented study of a non-selectable marker in paper V.

Probes can be labelled directly by radioactively labelled nucleotide analogs. As an example oligonucleotide probes can be labelled at the 3'end by using a terminal deoxynucleotidyltransferase and α-^{32}P CTP (a α-labelled deoxynucleotide triphospat dNTP) (Paper V). Radioactive labelling, although giving a high sensitivity, has several disadvantages related to safety and the lack of stability. Instead non-radioactive labelling can be used. In the study presented in paper V, dUPT coupled to an immunologically detectable hapten digoxigenin is used for 3'end labelling of the probe. After the hybridization to the probe, the target DNA undergoes a reaction with antibodies followed by a colour or luminescence reaction to ensure detection.

In situ colony hybridization is a useful method for the study of potential lateral gene transfer. Bacterial colonies are grown on a membrane placed on the agar plate. After *in situ* lysis the nucleic acids are bound to the membrane and later hybridized to the relevant probes (Grunstein & Hogness 1975). A control in form of a universal probe must be used to ensure that access to the cells have been made for correct interpretation of the results. The technique allows for the identification and isolation of the live colony bacteria.

More than one probe can be used on the same membrane. After a washing procedure a second hybridization reaction using a different probe can take place. Using a taxon specific probe for the subsequent hybridization would result in the identification of not only of the genetically modified gene but also the identification of the microorganism. Thus, in principle this method allows for the monitoring of loss or persistence of the modified gene as well potential lateral transfer of this gene.

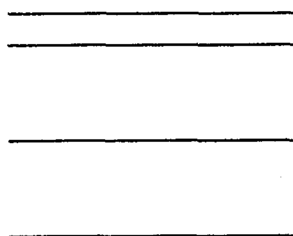
There are limitations to this method. Only culturable microorganisms can be identified by this method. This is not a problem when monitoring the original GMMO using polymerase chain reaction (PCR)-based methods (Saiki et al., 1988). The principle of the PCR-technique is the extraction of DNA from the sample. Specific DNA sequences from the sample are then amplified using specific primers and a polymerase. The basic reaction is repeated and PCR product accumulates (Figure 1). The sensitivity of the PCR method depends on the target DNA copy number, the nature of the primer and the purity of the DNA sample.

From being a qualitative method only, the PCR method has recently been modified to quantify DNA. This method is called competitive PCR or cPCR. A dilution series of an internal standard or competitor is amplified with an unknown concentration of the test DNA to be analysed. Due to the known concentration of the competitor DNA the estimate of test DNA can be made by comparison of amplification products by gel electrophoresis. This cPCR method has been used successfully for quantifying DNA from Pseudomonas from seawater (Leser 1995). The PCR based methods also have their limitations. They do not allow for the detection of lateral transfer.

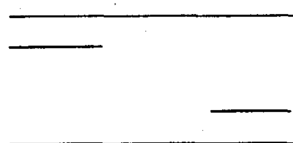
To qualify as a good marker in food related bacterial strains, it is essential, that the marker gene is not transferable. The marker should also not affect the viability of the microorganism. Special marker cassettes for insertion in bacteria have been made. They may have the disadvantage of instability and may be lost. The introduction of genetic material may also affect the viability of the microorganism. Instead a marker can be developed by introducing silent mutations. Such a mutation would not affect expression of the gene or the viability of the host microorganism. The specific mutation would allow for the use of nucleic acid based detection techniques and give the possibility of tracking the microorganism in complex microbiota. If such a mutation is situated on a plasmid, the potential lateral transfer can be detected.

A silent mutation type marker was designed. This was done, using *in vitro* mutagenesis of the proteinase gene PrtP gene of *Lactococcus lactis*. The nucleotides at the third position of three neighbouring codons were changed without altering the amino acid sequence. Specific probes for the modified (PLC2)and for the non-modified sequences (PLC1)

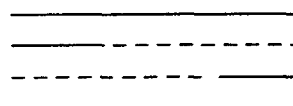
1. The double stranded target DNA is melted resulting in single stranded DNA



2. Primers are annealed to the target DNA



3. The DNA is extended by nucleotide addition from the primers by the action of the Taq polymerase in the presence of free deoxy-nucleotides



4. Repeating the steps 1-3 many times results in the exponential increase of target DNA.

Figure 1. The stages of PCR and the amplification of the DNA of the target organism.

were designed to detect the specific genes in hybridization studies (Hertel et al., 1992). The prtP variant gene was encoded on a plasmid. The stability of such a marker may increase by insertion into the chromosome (Pickup 1991). The probes, PLC1 and PLC2 for the variants of the proteinase gene were used in the investigation of possible lateral transfer described in paper V.

In the study described in paper V germ-free rats were associated with a proteinase deficient strain of *Lactococcus lactis,* Bu-2-60, to serve as a potential recipient. As donors in different *in vivo* conjugation experiments the following strains of *Lactococcus lactis* were used: MG1820 (pLMP1), MG1820 (pLMP1, pAMß1) and MG1820(pAMß1). *Lactococcus lactis* MG1820 (pLMP1) contained the plasmid with the modified proteinase gene. *Lactococcus lactis* MG1820 (pAMß1) containing the transferable plasmid pAMß1 served as a positive control for plasmid transfer in a similar experimental design. Finally *Lactococcus lactis* MG1820 (pLMP1, pAMß1) was made for the optimal transfer of both plasmids to recipient strain Bu-2-60. All of the used strain and plasmids could be individually identified by antibiotic resistance markers.

Faecal and later intestinal samples were analysed for the presence of donor, recipient and transconjugants by antibiotic resistance markers. Selected faecal and intestinal samples were also analysed for gene transfer of either the modified proteinase gene or the pAMß1 plasmid. This was done by colony hybridization with specific probes and with PCR. Transfer was observed for pAMß1 as an indication of possible transfer under the experimental conditions. No transfer of the modified proteinase gene prtP in the groups of gnotobiotic rats was observed using the classical antibiotic resistance screening or the molecular based methods (Paper V).

From these experiments it can be concluded that the silent mutation on the plasmid is not transferable and therefore suitable as a marker. The germ-free rat provided optimal conditions for the study of gene transfer and for the study of different identification methods. Looking at the efficiency of the different methods of detection, several comments have to be made. Regarding the detection limits:

- the method based on screening according to antibiotic resistance markers enables the detection of 10 cfu in one g faeces.

- the method based on PCR amplification and subsequent specific probe hybridization enables to detect $2x10^4$ cfu in one g faeces.

The traditional method based on a selective principle seems more useful than the molecular based method due to its simplicity and sensitivity. Progress has been made in the optimization of the PCR based methods. The use of a nested PCR approach allows the detection of 10^3 cfu in one g faeces (Paper V, Schleifer et al., 1995). In comparison it should also be noted, that using PCR does not allow for the association of the target with the living

cell. This is one advantage of the *in situ* colony hybridization. An interesting observation on *in vivo* transfer was obtained by this method and described in paper V. Since the result was obtained using conventional rats further comments on this method will follow in the next chapter.

9. STUDY OF BIOLOGICAL CONTAINMENT SYSTEMS AND ALTERNATIVE MARKERS USING ANTIBIOTIC TREATED CONVENTIONAL AND CONVENTIONAL RATS

As presented in chapter 4 several types of models of the mammalian gastrointestinal tract exist. Models using antibiotic treatment of animals has been developed to evaluate factors and mechanisms of potential health effects associated with environmentally released microorganisms. Due to the extensive use of antibiotics in therapy, a mouse model based on the effect of ampicillin has been used to study the influence of an antibiotic on the gastrointestinal microbiota exposed to environmental pseudomonads. The pseudomonads used in this study were commercially available strains used for PCB degradation. The model allowed for examination of indirect effects on the mouse. Such indirect effects may be the ability to colonise the gastrointestinal tract, competition with the resident microbiota and translocation of the Pseudomonas to organs (George et al., 1989).

Streptomycin treated mice have been used to elucidate *E. coli* colonisation. The initial studies using this model concentrated on the study of the colonisation capacity of different *E. coli*. The colonisation capacity of *E. coli* strains recently isolated from humans were compared to *E. coli* K12 strains (Cohen et al., 1979). Later this model has been used for several studies on the mechanisms of *E. coli* colonisation, including specific factors of colonisation like the formation of microcolonies (Cohen et al., 1990).

The use of streptomycin alters the ecology of the mouse caecum. Aerobic lactobacilli disappear and a simplification of microbiota is suggested to take place. This includes diminishing of the mucosal fusiform population, by that facilitating the association of pathogens with the intestinal mucosa (Hentges 1984, Hentges et al., 1990). Whereas streptomycin and ampicillin may cause major changes in the gastrointestinal microbiota of man and animal, the antimicrobial agent norfloxacin has the advantage of reducing the group of Enterobacteriaceae leaving the anaerobic microbiota unaffected (Edlund et al., 1987).

Norfloxacin has been used for selective decontamination of pigs (Van der Waaij 1989). The norfloxacin treated pig was later used as an *in vivo* model for testing the colonisation capacity of *E. coli* (Nielsen & Schlundt 1992). Norfloxacin treatment of rats has proven useful for a similar reduction of CR by temporal elimination of Enterobacteriacea. This makes it possible to study survival and colonisation of specific *E. coli* strains (Schlundt et al., 1992).

Norfloxacin treated conventional rats have also been used in the studies of the biological containment system based on the random activation of the *gef*-gene presented in chapter 5. The results from the study of *E. coli* with the *gef*-gene in the gastrointestinal tract of norfloxacin treated rats are presented

in Appendix A II. In the rats dosed with *E. coli* with the *gef*-gene (BJ19/pSM910), the concentrations in faecal samples remained stable at 10^7 cfu/g faeces. This is almost in the same order of magnitude as normally found in untreated rats (10^6 cfu /g faeces to 10^7 cfu /g faeces). Simultaneous dosing of BJ19/pSM910 and wildtype *E. coli* BJ4 did not alter the results observed with BJ19/pSM910 alone: BJ19/pSM910 was found in concentrations of 10^7 cfu/g faeces throughout the experimental period. No reductions in *E. coli* BJ19/pSM910 concentrations as a consequence of *gef*-gene induction were thus observed. This observation was in concordance with the results obtained by simultaneous dosing of germ-free rats described earlier in chapter 5 even though the bacterial concentrations were not of the same magnitude.

The result from the norfloxacin treated conventional rats support the hypothesis presented in chapter 5. The intra species competition with a resident microbiota is the most important factor leading to the competitive exclusion of *gef*-gene containing *E. coli.* The difference in colonisation capacity, illustrated by *E. coli* concentrations of 10^{10}cfu/g faeces observed in gnotobiotic rats to 10^7 cfu/g faeces observed in the norfloxacin rats, can be explained by the presence of an otherwise "normal" microbiota in the norfloxacin treated rats. The reduced capacity may be seen as reduced number of attachment sites/residential niches and limitations in nutrients due to the numerous specialised microorganisms constituting the "normal" microbiota. The leftover possibilities reflect the possibilities normally given to natural *E. coli* population.

Conventional rats have also been exposed to the *E. coli* strains used in the studies presented in paper II and in the above discussed study using the norfloxacin treated rats. The results from the studies with *E. coli* strains with and without the *gef*-gene in conventional are presented in Appendix A III. In all the rats the dosed *E. coli* strains rapidly disappeared from the faecal samples and were no longer detected after day 6. The reduction observed in the concentrations of *E. coli* in faecal samples were almost identical for the 3 different strains and seems independent of genetic status. Thus, no difference in reduction could be related to the presence of the *gef*-gene. This result is in concordance with the hypothesis set out in chapter 5. The presence of an established *E. coli* containing microbiota reduces the possibility of colonisation. This results in the wash out of exogenous *E. coli* strains irrespective of expression of a *gef*-gene. This colonisation resistance (CR) exerted by the normal microbiota is not only working against the Enterobacteriacea. CR in principle work against all exogenous microorganisms although some pathogens may overcome this important protection against infection.

Dosing conventional rats with the *Lactococcus lactic* used in the study presented in paper V also lead to the rapid wash out of the *Lactococcus lactis* strains. Similar results have been obtained from the human gastrointestinal tract using a genetically marked *Lactococcus lactis* strain. The strain was marked by inserting a gene coding for nisin (*nis*A) together

with chromosomally located antibiotic resistance marker. The study showed that the *Lactococcus* strain was killed during the gastrointestinal passage. One per cent of the total numbers of consumed cells could be recovered only in a period of three days after administration (Klijn et al., 1995). This indicates that the *Lactococcus* strains are not capable of colonising the gastrointestinal tract. This will however be to general a statement to make for all *Lactococcus* strains.

The purpose of the study presented in paper V was to find out if lateral transfer of the used *Lactococcus lactis* plasmids pLMP1 and pAMß1 could take place in the mammalian intestine. No lateral transfer of pLMP1 was observed in the gnotobiotic rats. Lateral transfer of the conjugative broad host range plasmid pAMß1 occurred at high rates. This resulted in colonisation of the gastrointestinal tract by transconjugants (paper V, chapter 8). Lateral transfer of pAMß1 from *Lactococcus lactis* to *Enterococcus* from the endogenous microbiota was also observed in one conventional rat. The transfer was detected by the routine plating of faecal samples on selective agar supplemented with the relevant antibiotics. The identity of the plasmid and the receiving microorganism were analysed using specific hybridisation probes. Using this technique, the recipient could be characterised as *Enterococcus faecalis* or its close relative.

The finding is important. The earlier findings by Duval-Iflah of gene transfer from transient *Serratia liquefaciens* to resident *E. coli* (Duval-Iflah et al., 1980) and the findings of our study of plasmid transfer between *Lactococcus lactis* strains (paper IV) confirm the hypothesis, that even if the GMMO is not capable of colonising the gastrointestinal tract the modified genetic material may be laterally transferred to the endogenous microbiota. In relation to this discussion of gene transfer it may be of interest, that it has been shown, that specific marker genes extracted from human faecal samples could be detected by PCR up to four days after administration of the marked *Lactococcus lactis* strain. This detection of specific genes was possible though no viable marked cells were present in the samples. This observation may be explained by the simple difference in sensitivity of the detection or, as suggested by the authors, by the presence of nonculturable marked cells or even DNA from lysed cells (Klijn et al., 1995).

The colony hybridization technique contributed to the finding of this transfer to the normal microbiota. As pointed out in chapter 8 this technique allows for the identification of a living recipient even among thousands of microorganisms. There are of course limitations to this technique. Only culturable microorganisms can be detected. Using a selective media it was possible to analyse 10^4 small colonies on the membranes. This should be related to the fact that the selected Lactococcus population is only a part of a total population. In comparison the total population of bacteria in caecal samples from a rat is approximately 10^{11}/ g (Mallet et al., 1987).

The overall conclusion of the studies in the norfloxacin treated and conventional rat is, that they are useful models in the elucidation of the interaction of the GMMO with the "normal" microbiota. The effect of

special groups of bacteria and gene transfer can be studied using these models.

10. EVALUATION AND PERSPECTIVES

Major differences exist between animals and humans regarding microbiota composition and metabolism, and data obtained from animal models should be interpreted carefully. This must always be considered when extrapolating from animal to man. Also in designing the animal studies the scientist must be aware of factors that may influence the outcome of the study. Stress due to transportation, the use of toxic bedding are well-known examples from animal science of sources leading to misinterpretation of results. Several factors are known to influence the gastrointestinal microbiota of animals and man. Stress leads to the production of several hormones including ACTH-corticoid adrenalin and noradrenaline. The hormones are known to influence physiology and behaviour (Tuli et al., 1995) and could also change the intestinal microbiota. Studies of stress provoked by crowding or heat have shown to lead to changes in the microbiota of the rat. *Staphylococci*, *Streptococcus*, *Corynebacterium* and Enterobacteriacea were generally increased by stress. Severe stress lead to an increase in *Bacteroides* and the decrease of *Lactobacillus*. Bodyweight and feed effiencies were markedly reduced under these stress conditions (Suzuki et al., 1989). Therefore in animal experiments it is important to let the animals acclimatize after transportation, reduce the stress caused by animal technicians etc.. Fulfilment of stress reduction have been important elements in the presented studies.

Another very important element in the design of the studies has been the uniformity of the experimental conditions. All experiments have been made in isolators. The use of isolators is of course a prerequisite for working with gnotobiotes. The isolators along with the standard procedures of decontamination and sterilisation provide protection against foreign microorganisms. This is a benefit for all studies including studies with the conventional rats and norfloxacin treated rats. The interpretation of results does not have to take the possibility of the exogenous factors in consideration. On the other hand long term stay in isolators may lead to alteration of the microbiota as reflected in MAC parameters (Norin et al., 1993). The studies presented using conventional rats have an experimental period of less than a month and are not considered subjected to the described long-term changes. A special isolator setup developed at the Institute of Toxicology has made it possible to change the pressure of the isolators from the high pressure, normally used when working with germ-free rats, to a pressure less than the environment, when working with the GMMO. The low pressure together with HEPA filters ensures the protection of the environment and the safety of the laboratory technicians as required by the Danish authorities.

The rat exerts coprophagy. The animal excretes special soft pellets which are delivered directly to the mouth. Coprophagy is of nutritional importance. The requirements of special vitamins like B and K vitamins are influenced by the microbiota (McBee 1977). In our studies we have not noticed any effects like "re-administration" of bacteria, which could be related to coprophagy.

The rats used in the presented studies have been on a so-called breeding diet. Such a diet contains sufficient amounts of nutrients. The original high nutritional quality of diet and the use of sterilisation by radiation (in contrast to autoclaving) may be responsible of the good physiological conditions of the germ-free rats and the good breeding results obtained at the Institute of Toxicology.

The use of animal models overcome the limitations using man. Studies can be performed that would not be possible on ethical grounds in humans. In order to compare the used animal models some of the results presented in appendix A using *E. coli* BJ19/pSM910 are summarised in figure 2. A single

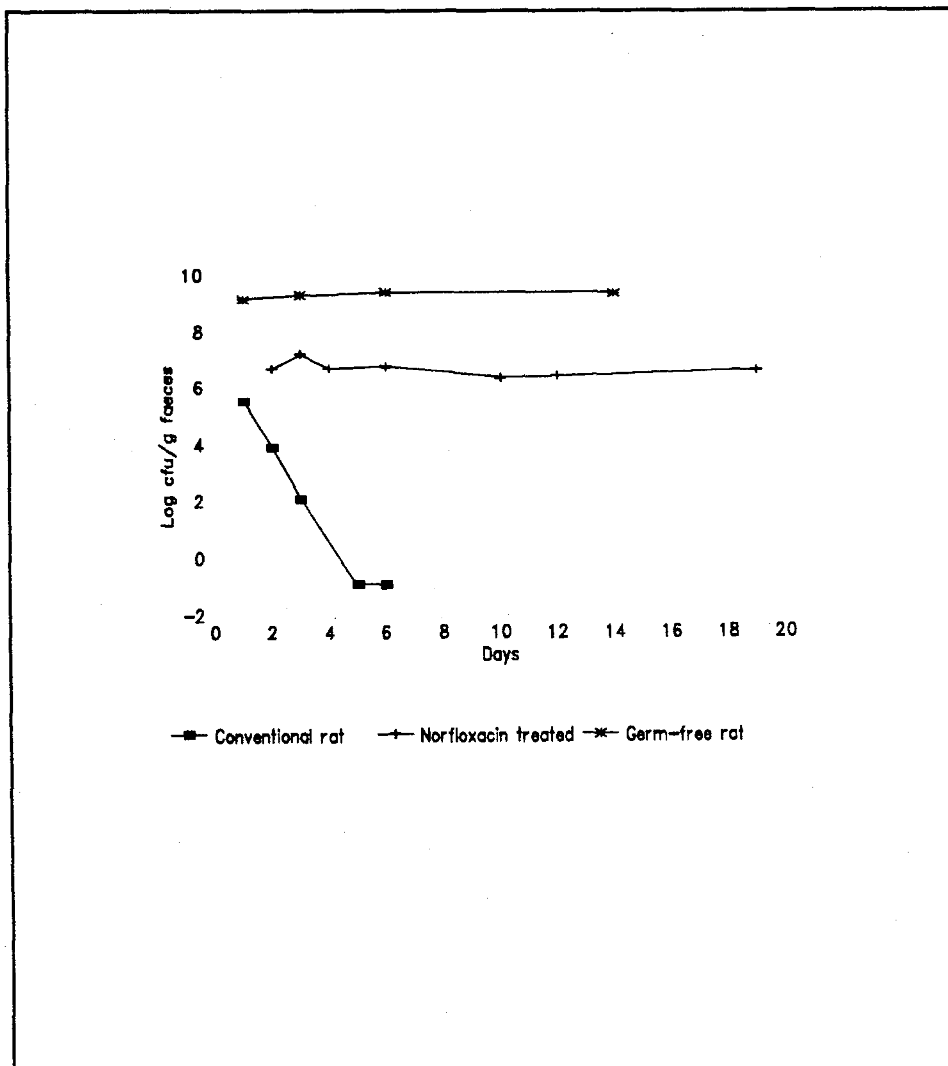


Figure 2. The fate of BJ19/pSM910 administered to a conventional rat, a norfloxacin treated conventional rat and a germ-free rat. Concentrations of *E. coli*/pSM910 in faecal samples were determined by plating on MacConkey agar supplemented with nalidixin and kanamycin relating to the antibiotic resistance markers of BJ19/pSM910.

high dose of *E. coli* BJ19/pSM910 was given to conventional rats, norfloxacin treated conventional rats and germ-free rats. After dosing faecal concentrations of *E. coli* BJ19/pSM910 were determined. Characteristic differences in colonisation pattern of *E. coli* BJ19/pSM910 related to the animal models were present.

With this figure in mind some experimental advantages of the germ-free rat can be summarised:

- The germ-free rat constitutes a well-defined system with intact biological parameters.

- The germ-free rat makes long term colonisation in high concentrations with a specific bacteria possible.

- The colonisation of germ-free rats in high numbers of specific bacteria makes it possible to study gene transfer even at very low frequencies.

- The germ-free rat makes it possible to study the potential biological effect of a product coded for by inserted genes in GMMO under long term exposure.

- The germ-free rat may be conventionalised

- The germ-free rat can be associated with a human derived microbiota.

The well-defined system with intact biological parameters is to be seen in contrast to *in vitro* models, which do not have the capability of giving host related biological responses. The conventional and norfloxacin treated rat are capable of such biological host related responses, but do not have the well-defined microbiota. This could make it difficult to relate a host response to a particular microorganism. The influence of an existing microbiota may also affect the study of the effect of a gene on the microorganism it self. If one wanted to study the effect of the *gef*-gene in *E. coli* BJ19/pSM910 in the conventional rat it would be difficult to estimate an effect. The rapid wash out would also make studies of potential gene transfer difficult.

The use of norfloxacin treated rats provide better possibilities for the study of long-term colonisation even though it is not in high concentrations. The disadvantage lies in that only *E. coli* of high colonisation potential will colonise the norfloxacin treated rat. The *E. coli* strain used in studies presented in paper III was a K12 derived strain with limited colonisation potential so it only colonised the germ-free rat (unpublished results). Finally the norfloxacin treated rat primarily allow for the introduction of test bacteria belonging to the Enterobacteriacea. The norfloxacin treated rat compared to the streptomycin treated mice has several advantages. The norfloxacin treated rat only needs a temporary treatment with the antibiotic. No selective pressure on a streptomycin resistant test microorganism is

exerted as in the streptomycin treated mouse by the required continuous use of streptomycin in the drinking water. The relative disturbance of the microbiota is also considered to be greater in the streptomycin treated mouse than in the norfloxacin treated rat. Several bacterial groups are affected by streptomycin in contrast to selective decontamination of the Enterobacteriacea by norfloxacin.

The germ-free rat also makes it possible to study the effect of a GMMO in the interaction with a normal microbiota. Earlier studies with gnotobiotic mice showed that it was possible to keep an associated *E. coli* strain in the mice together with the microbiota. If the *E. coli* was administered first, no wash out was seen when the normal microbiota was established (Freter 1978). A study from the Karolinska Institute in Sweden has shown, that conventionalisation in the form of establishment of a biochemically active intestinal system may take place by simply housing the germ-free rat with a conventional rat (Midtvedt et al., 1987). Conventionalisation may involve the risk of disease among the animals. The use of SPF rats reduces this risk. By first monoassociating a germ-free rat with a marked *E. coli* followed by caging with a conventional rat, results comparable to those in mice were obtained at the Institute of Toxicology (Jacobsen 1992).

The introduction of a microbiota into the germ-free rat is not restricted to the association with a rat microbiota. The germ-free rat has a great scientific potential in the association with a human faecal microbiota, the so-called *h*uman *f*aecal flora *a*ssociated (hfa) rat. Several studies have revealed the successful insertion of a human microbiota in rodents and its usefulness in studies of the interaction of the microbiota with specific bacterial groups like the Bifidobacteria (Hirayama et al., 1991). The hfa-rat has already been of practical use in studies of the effect of diet on the human gastrointestinal microbiota (Mallet et al., 1987). The hfa-rodent is recommended as a useful model for the study of metabolic activities, the study of toxicity of chemicals and gastrointestinal infections (Rumney & Rowland 1992). The use of the hfa-rat makes the extrapolation from animal to man easier. The disadvantage is the relatively high cost of the germ-free rat and maintenance of the hfa-status using isolators.

The experimental advantages presented above may lead to the assumption that the germ-free rat is the only and superior model for the testing of GMMO. This would be the wrong conclusion to draw. All of the presented animal models are scientifically useful. The use of a particular animal model will always depend on the questions to be answered. As pointed out in chapter IV the germ-free rat may be regarded as a "worst case model" simulating a situation of no protective microbiota. Interaction with the normal microbiota and gene transfer like the "random, natural" gene transfer from *Lactococcus lactis* to the *Enterococcus faecalis* like bacteria involves the use of conventional rats. The use of norfloxacin rats added knowledge on the influence of the complementary microbiota on the possible exclusion of *E. coli* BJ19/pSM910.

Risk evaluation of GMMO in relation to human health effects includes the

answering of several questions regarding the GMMO relating to its ability to cause disease, to produce toxins, to colonise, the effect of new gene product or the use of antibiotic resistance genes as markers (Knudsen 1987). Looking at these points, the use of the germ-free animal has clearly contributed to the elucidation of some of the presented questions.

In conclusion: The use of germ-free rats has successfully resulted in information regarding the survival and colonisation of GMMO as well as gene transfer between GMMO and microorganisms in the mammalian gastrointestinal tract. Some of this information would have been impossible or difficult to obtain using conventional rats. Finally - The use of germ-free rats has lead to a significant reduction in the number of laboratory animals to obtain the above mentioned information. The future will focus on the effect of microorganisms, including effects of GMMO. Probiotics, starter cultures and novel foods of microbiological origin will find their way to the market. This will raise the question how to test for their potential adverse effects to the human health effect including effect on the host immune system. The germ-free rat and the human faecal flora associated rat will be an obvious choice for such studies.

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APPENDIX A: STUDY OF A BIOLOGICAL CONTAINMENT SYSTEM IN CONVENTIONAL, NORFLOXACIN TREATED CONVENTIONAL AND GERM-FREE RATS.

DATA FROM EXPERIMENTS WITH A BIOLOGICAL CONTAINMENT SYSTEM BASED ON THE ACTIVATION OF THE GEF GENE IN CONVENTIONAL, NORFLOXACIN TREATED AND GERM-FREE RATS

INTRODUCTION

The *gef* gene belongs to the same family of killing genes as *hok*, both coding for proteins lethal to *E. coli*. Using germ-free rats and *E. coli* as test bacteria we have studied genetic constructions made for high biological containment. The constructions were based on the principle of activation of an inserted gene coding for a protein lethal to *E. coli* (Paper I, II). The effect of the *gef* gene is shown to be a shorter decimation time, T_{90} of *E. coli* in a competition situation (Paper II).

This appendix comprises the results of experiments with the same bacterial strains as used in paper II, but using different animal models/and or a different experimental design.
The results presented are from the study of:

- the fate of *E. coli* with and without the *gef* gene in conventional rats (experiment I)

- the fate of *E. coli* with the *gef* gene in norfloxacin treated rats (experiment II)

- the simultaneous competition between *E. coli* with the *gef* gene and *E. coli* BJ4 in norfloxacin treated rats (experiment II)

- the simultaneous competition between *E. coli* with the *gef* gene and *E. coli* BJ4 in germ-free rats (experiment III)

The overall purpose of this experiment was to relate the results obtained in germ-free rats with results obtained from conventional rats. The discussion of the results is presented in chapter 6 and 8.

MATERIALS AND METHODS

Animals

The animals used in the experiments presented in appendix A had identical experimental conditions. All rats were kept in separate flexible isolators (Isotec type 12234, Olac Oxford, UK). The rats were given 50 kGy-irradiated Altromin 1314 (Brogaarden Gentofte, DK) and 50 kGy-irradiated drinking water *ad libitum*. All rats were housed individually in type III macrolon cages supplied with a grid and 50 kGy-irradiated hardwood bedding (Red Special, Spanwell). The isolators had a negative pressure during the experiment, in order to avoid unintentional release of the GMMOs. Air-inlets and -exhausts were supplied with Hepa filters (Interfilta limited, grade IFO). The ambient room temperature was maintained at 20±1°C with a light period from 9.00 p.m. to 9.00 a.m. The relative humidity was approximately 55±5%. Air was changed in each isolator between eight and ten times per hour.

Bacteria and plasmids

E. coli BJ4, a wildtype *E. coli* isolated from a Wistar rat at the Institute of Toxicology (Jacobsen 1992). *E. coli* BJ19, a selected nalidixin resistant mutant of BJ4 (Nielsen & Schlundt 1992). Plasmids were kindly supplied by S. Molin, The Technical University of Denmark. Plasmid pSM910 contains switch-*gef*, *fim*B and *fim* E, producing the *fim*B gene product in surplus (Klemm et al., 1995), pSM1020 contains only the switch (S. Molin, personal communication). Both plasmids are Km^r and were separately transformed into BJ19. Transformation was done according to Sambrooke et al., (1989). Bacterial cultures used for dosing were grown overnight at 37°C. The stationary phase cultures were centrifuged at 5000 *g* for 5 min and resuspended in physiological saline with 0.1 per cent peptone (Oxoid).

Bacteriological quantification

Serial dilutions of faecal samples taken from the rectum were made using physiological saline with 0.1 per cent peptone (Oxoid). The bacterial concentrations of *E. coli* were estimated using the spread plate method and MacConkey agar (Oxoid CM115) supplemented with the relevant antibiotics in the following concentrations: Kanamycin (Sigma K1377) 50µg/ml, nalidixin (Sigma N4382) 40µg/ml.

Statistical analysis

Bacterial counts were transformed to $\log_{10}$ values. Calculations were done, using QuattroPro 4.0, Borland Inc. 1992.

A I. THE FATE OF *E. coli* WITH AN WITHOUT THE *gef* GENE IN CONVENTIONAL RATS.

This experiment was carried out in order to elucidate and compare the passage time of different *E. coli* with and without the biological containment system based on the random activation of the *gef* gene. The same constructions were used as presented in paper II.

Animals (See Materials and methods)
A total of 30 Mol:Wist rats (200-250 g) were used. The rats were divided into 3 groups of 5 males and 5 females.

Experimental procedure
The rats were given by gavage p.o. 1 ml bacterial culture according to Table 1.

Table A I- 1: Dosing of the animals.

Group	No. rats	Bacteria/plasmid	Dosis (cfu/ml)
A	10	BJ19/pSM910	Approx. $3x10^8$
B	10	BJ19	Approx. $2x10^8$
C	10	BJ19/pSM1020	Approx. $4x10^8$

Faecal samples were taken through the experimental period of 8 days.

Results

Table AI-2: Concentrations ($\log_{10}$ cfu/g faeces) of *E. coli* strains in faecal samples

Group	Day	BJ19/pSM910		Total *E. coli*
	1	4.94±2.04	6.26±0.58	
	2	2.07±2.09	6.43±0.50	
A	3	0.63±1.86	6.26±0.67	
	6	-0.29±1.43		ND
	7	-0.49±0.96	6.19±0.48	
	8	-0.78±0.63		ND
		BJ19		Total *E. coli*
	1	4.48±2.00	5.73±0.56	
	2	1.55±1.68	5.85±0.42	
B	3	-0.52±0.98	5.96±0.60	
	7	-0.53±1.41	5.60±0.45	
	8	-0.43±1.50	6.41±0.72	
		BJ19/pSM1020		Total *E. coli*
	1	5.33±0.21	6.73±0.55	
	2	2.47±2.14	ND	
C	3	1.01±2.15	5.84328	
	7	-1±0		6.53±0.359
	8	-0.53± 1.25	6.25±0.3	

ND= not determined

In all the rats the dosed *E. coli* strains in average are no longer detected after day 6. The reduction observed in the concentrations of *E. coli* in faecal samples are almost identical for the 3 different strains.

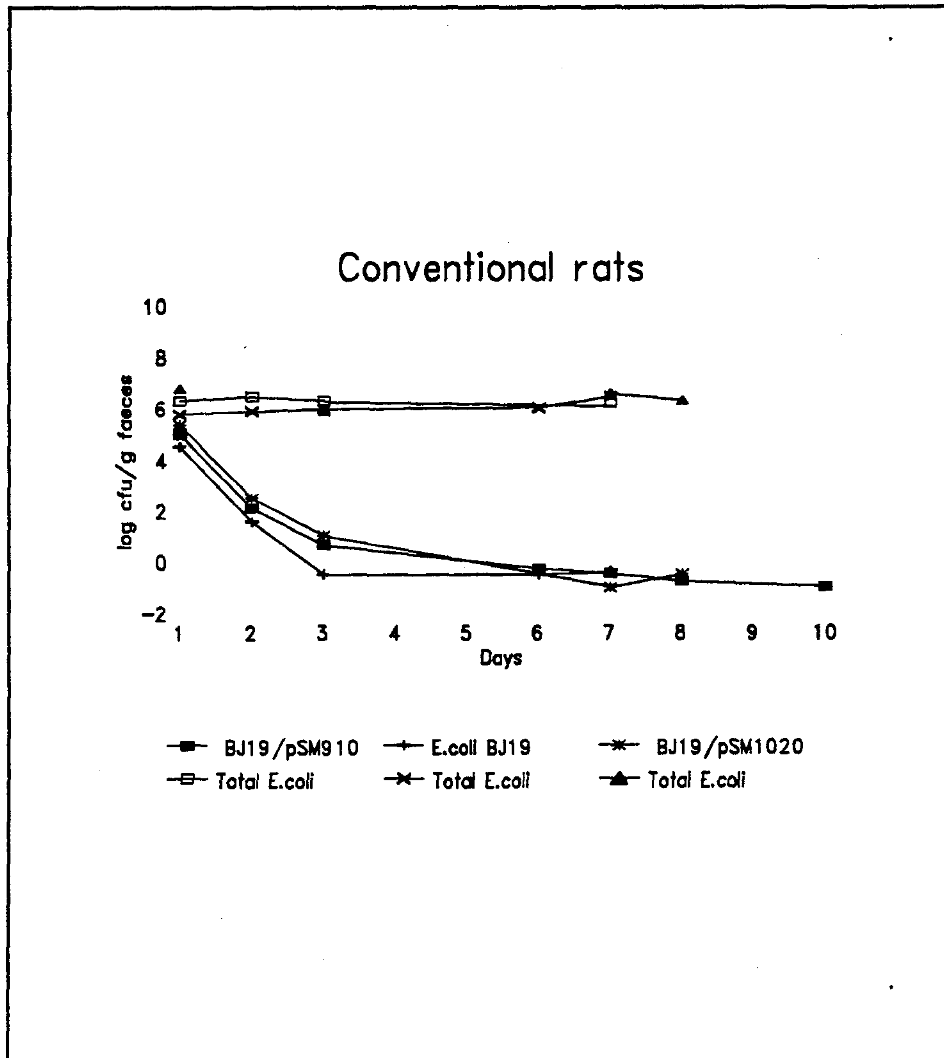

Figure A-1. *E. coli* concentrations in faecal samples from 30 conventional rats divided into 3 groups. Group A rats were dosed with approximately $3x10^8$ cfu of *E. coli* BJ19/pSM910 on day 0. Group B rats were dosed with approximately $4x10^8$ cfu of *E. coli* BJ19/pSM1020 on day 0. Group C rats were dosed with approximately $2x10^8$ cfu of *E. coli* BJ19 on day 0. *E. coli* BJ 19 is a nalidixin resistant BJ4 derivative. *E. coli* BJ 19 /pSM910 contains the plasmid pSM910 with *gef*-gene and the gene coding for the resistance to kanamycin. *E. coli* BJ 19 /pSM1020 contains the plasmid pSM1020 with the gene coding for the resistance to kanamycin but not the *gef*-gene The *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with nalidixin, and nalidixin and kanamycin. Vertical line represents standard deviations. The detection limit is 10^1 cfu/g faeces.

A II. THE FATE OF *E. coli* WITH THE *gef* GENE IN NORFLOXACIN TREATED CONVENTIONAL RATS.

Treatment with the antibiotic norfloxacin results in the selective elimination of Enterobacteriacea. Upon the successful elimination of Enterobacteriaceae the test strains were given to the treated rats. Group A rats were given E. coli with the gef gene (BJ19/pSM910). Group B rats were given a mixture of E. coli BJ19/pSM910 and E. coli BJ4.

Animals (See also Materials and methods)
A total of 10 male Mol:Wist rats (150 g) were used. The rats were divided into 2 groups.

Bacteria and plasmids (See also Materials and methods)
The mixed bacterial culture of *E. coli* BJ19/pSm910 and *E. coli* BJ4 was made from 5 ml of overnight cultures. The cultures were centrifuged 5000 rpm. The pellets were resuspended in approximately 3 ml. The two cultures were mixed resulting in a total volume of approximately 6 ml.

Experimental procedure
The rats were pretreated with 25 mg norfloxacin /kg bodyweight (Zoroxcin: Merck, Sharp & Dohme) p.o. by gavage for 3 days prior to the experimental period. The treatment was repeated using a dose of 40 mg/kg bodyweight of norfloxacin due to unsuccessful elimination of Enterobacteriacea in all animals. The repeated treatment resulted in the expected elimination as no *E. coli* was observed on MacConkey plates. The rats were given by gavage p.o. 1 ml bacterial culture according to Table 1.

Table A II-1. Dosing of the animals

Group	No. rats	Bacteria/plasmid	Dosis (cfu/ml)
A	10	BJ19/pSM910	Approx. $3.1x10^8$
B	10	BJ4 + BJ19/pSM910	Approx. $7x10^8$

Faecal samples were taken throughout the experimental period of 4 weeks.

Results

Table A II-2: Concentrations (log_{10} cfu/g faeces) of *E. coli* strains in faecal samples of rats in group A.

Day	Total *E. coli*	BJ19/pSM910
2	6.62±0.25	6.60±0.28
3	7.10±0.43	7.09±0.41
4	6.38±0.67	6.74±0.32
6	6.49±0.28	6.37±0.40
10	6.88±0.45	6.60±0.53
12	7.45±0.31	6.20±0.41
19	6.98±0.26	6.26±0.34
24	6.75±0.41	3.39±3.60*)

*) Nalidixin and kanamycin resistant *E. coli* were not found in 2 rats.

Table A II-3: Concentrations (log_{10} cfu/g faeces) of *E. coli* strains in faecal samples of rats in group B.

Day	Total *E. coli*	BJ19/pSM910
2	6.74±0.51	6.63±o.49
3	6.18±0.50	6.05±0.56
4	6.48±0.65	6.50±0.34
6	7.11±0.60	7.06±0.63
10	7.34±0.90	7.29±0.74
12	7.25±0.55	7.20±0.35
19	6.42±1.09	3.39±3.68*)
24	6.27±0.79	4.15±2.72#)

*) Nalidixin and kanamycin resistant *E. coli* were not found in 2 rats.
#) Nalidixin and kanamycin resistant *E. coli* were not found in 1 rat.

The results of group A faecal samples shows that the concentration of total *E. coli* is approximately 10^7 cfu/g faeces throughout the experimental period. The concentrations of the *gef*-gene containing *E. coli* represented by the cfu/g faeces of the nalidixin and kanamycin resistant *E. coli* are almost identical to the total *E. coli* counts. Even though there are minor difference compared to the total *E. coli* concentrations in the faecal samples, the BJ19/pSM910 concentrations remains stable and in the same order of magnitude. Late in the experimental period no nalidixin and kanamycin resistant *E. coli* were found in two rats, indicating the disappearance of BJ19/pSM910.

The results of group B faecal samples are almost identical to the results of group A samples. The concentrations of total *E. coli* are approximately 10^6-10^7 cfu/g faeces. The group B results also show a marked reduction in the concentration of BJ19/pSM910 in the last week of the experimental period, again indicating the disappearance of the test bacteria.

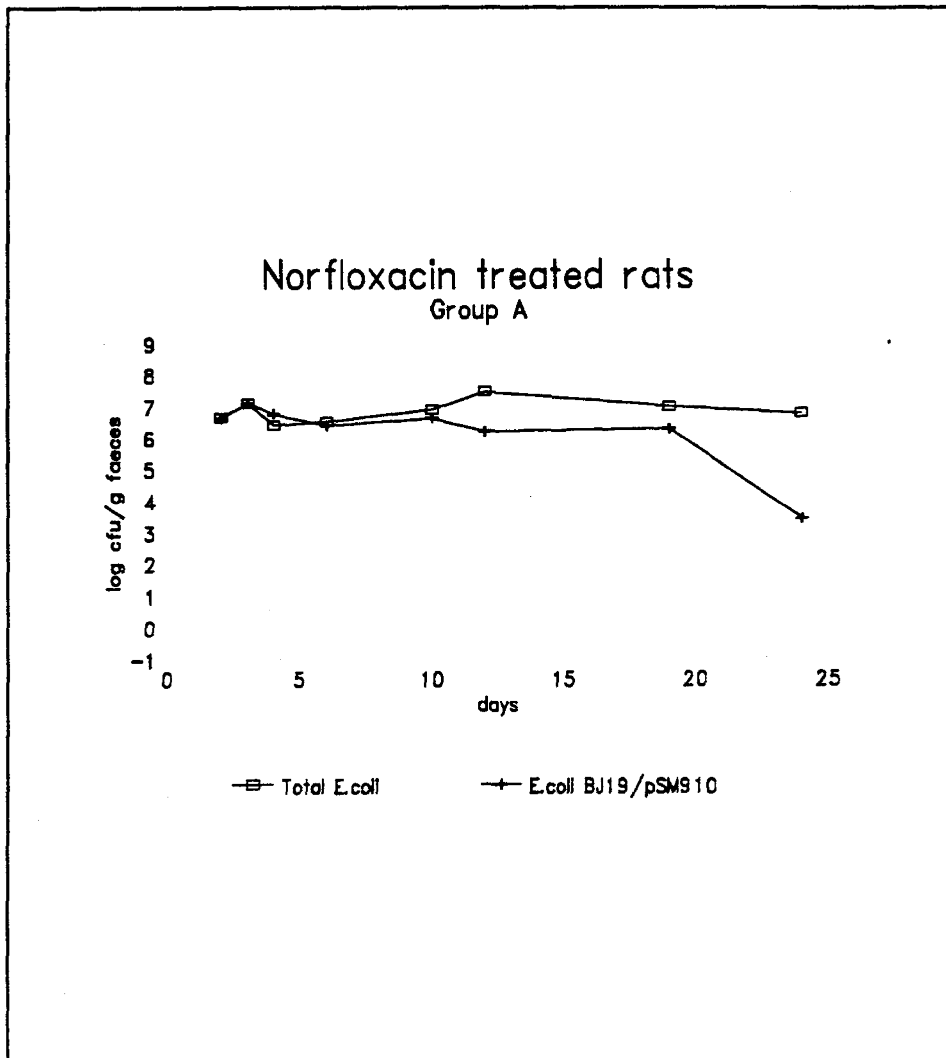

Figure A-2. *E. coli* concentrations in faecal samples from norfloxacin treated conventional rats. 5 male rats were dosed with approximately 3.1×10^8 cfu of *E. coli* BJ19/pSM910 on day 0. *E. coli* BJ19 /pSM910 is a BJ4 derivative containing the plasmid pSM910 with *gef*-gene and the gene coding for the resistance to kanamycin. The *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with nalidixin and kanamycin. Vertical line represents standard deviations. The detection limit is 10^1 cfu/g faeces.

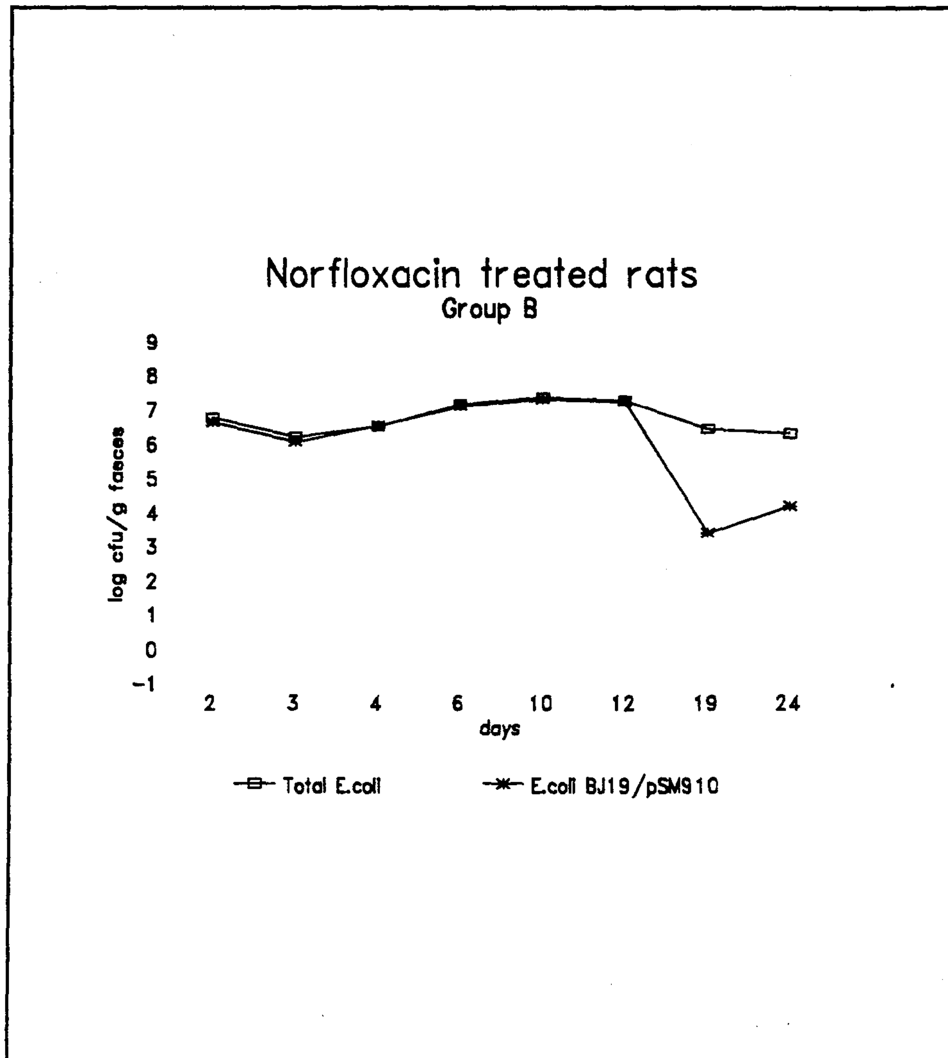

Figure A-3. *E. coli* concentrations in faecal samples from norfloxacin conventional treated rats. 5 male rats were dosed simultaneously with *E. coli* BJ4 and *E. coli* BJ19/pSM910 using a mixed culture of approximately $7x10^8$ cfu on day 0. BJ4 is a wildtype *E. coli* strain and sensitive to the antibiotics nalidixin and kanamycin, *E. coli* BJ19 /pSM910 is a BJ4 derivative containing the plasmid pSM910 with *gef*-gene and the gene coding for the resistance to kanamycin. The *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with nalidixin and kanamycin. Vertical line represents standard deviations. The detection limit is 10^1 cfu/g faeces.

III. THE COMPETITION OF *E. coli* WITH THE *gef* GENE AND WILDTYPE *E. coli* BJ4 IN GERM-FREE RATS.

The germ-free rats were given a mixture of E. coli BJ19/pSM910 and *E. coli* BJ4. This experimental design gives equal conditions of competition for all the strains given to the rats.

Animals (See also Materials and methods)
A total of 4 male germ-free rats (600-700g) were used.

Bacteria and plasmids (See also Materials and methods)
The mixed bacterial culture of *E. coli* BJ19/pSM910 and *E. coli* BJ4 was made from 5 ml of overnight cultures. The cultures were centrifuged 5000 rpm. The pellets were resuspended in approximately 2.5 ml. The two cultures were mixed resulting in a total volume of approximately 5 ml.

Experimental procedure
The rats were given by gavage p.o. 1 ml bacterial culture resulting in a dose of approximately $5x10^8$ c.f.u./rat. Faecal samples were taken throughout the experimental period of 4 weeks.

Results

Table A.III-1. Concentrations (log_{10} cfu/g faeces) of *E. coli* strains in faecal samples from gnotobiotic rats.

Day	Total *E. coli* cfu/g faeces	Nal^r *E. coli* cfu/g faeces	Kan^r,Nal^r *E. coli* cfu/g faeces
1	-	-	-
2	9.35±0.11	8.88±0.11	8.95±0.13
3	9.41±0.27	8.71±0.27	8.78±0.30
6	9.39±0.10	8.98±0.10	9.00±0.20
8	9.58±0.12	9.23±0.12	9.45±0.37
10	9.76±0.13	9.26±0.13	9.23±0.09
13	9.53±0.24	9.07±0.24	9.23±0.08
23	9.71±0.16	9.54±0.16	9.21±0.09
27	9.32±0.15	9.27±0.15	9.04±0.09

The total *E. coli* concentration is high approximately 10^9cfu/g faeces. The total number of *E. coli* remains stable throughout the experimental period. The nalidixin resistant *E. coli* values are almost identical. Finally the concentration of kanamycin and nalidixin resistant *E. coli* resembling *E. coli* BJ19/pSM910 is high approximately 10^9cfu/g faeces and remains stable throughout the experimental period.

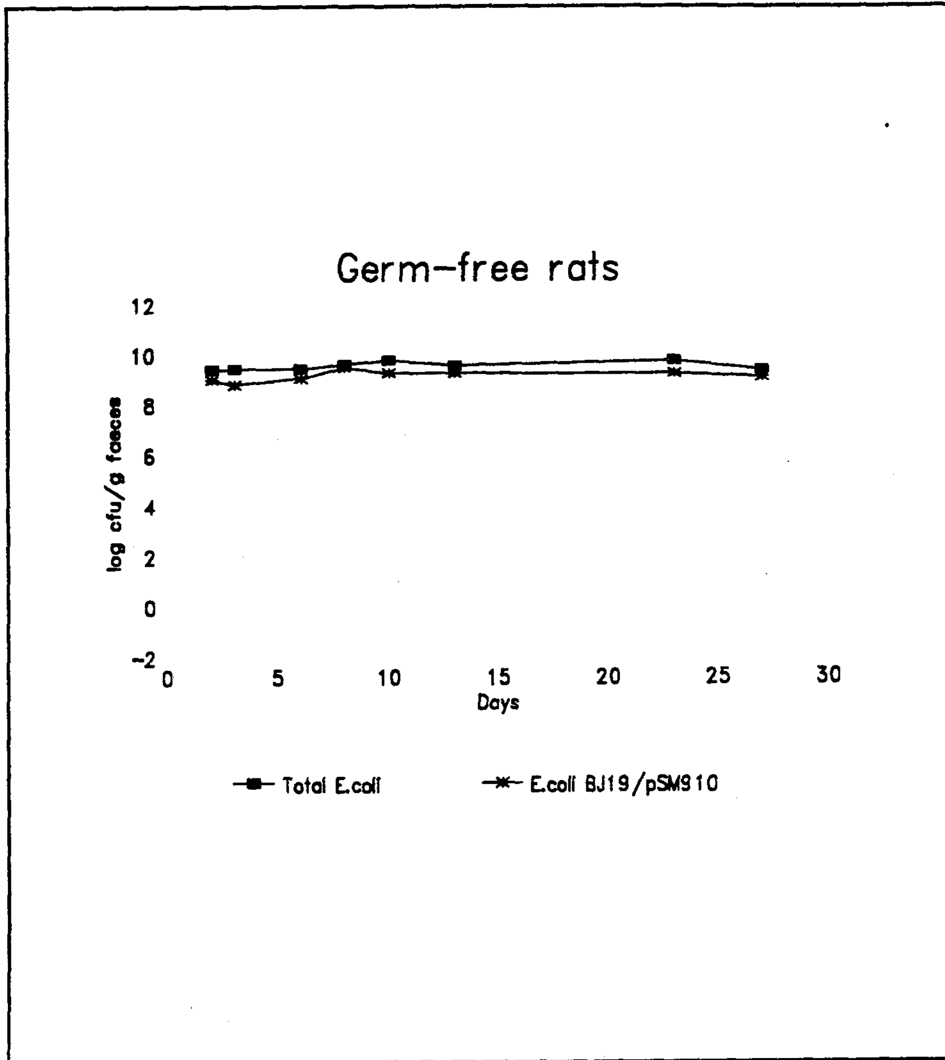

Figure A-4 *E. coli* concentrations in faecal samples from gnotobiotic rats. Four germ-free male rats were associated simultaneously with *E. coli* BJ4 and *E. coli* BJ19/pSM910 on day 0. BJ4 is a wildtype *E. coli* strain and sensitive to the antibiotics nalidixin and kanamycin, *E. coli* BJ19/pSM910 is a BJ4 derivative containing the plasmid pSM910 with *gef*-gene and the gene coding for the resistance to kanamycin. The *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with nalidixin and kanamycin. Vertical line represents standard deviations. The detection limit is 10^1 cfu/g faeces.

APPENDIX B: SHORT INTRODUCTION TO GNOTOBIOLOGY

Terminology:

Gnotobiology is derived from the greek words *gnotos* and *biota,* meaning known flora or fauna. A gnotobiot is an animal with a known microbial flora or microbiota.

Germ-free	A gnotobiot free from any other detectable form of life.
Mono-associated	A gnotobiot with one microorganism of known identity.
Di-associated	A gnotobiot with two microorganisms of known identity.
Poly-associated	A gnotobiot with many microorganisms of known identity.
SPF	Animals free from specified pathogens, but otherwise with an undefined microbiota.
Conventional	Animals with a "normal" but undefined microbiota.

APPENDIX C: GLOSSARY

Allochthonous	Foreign to a particular habitat
Allogenic factors	Regulatory forces exerted by the host animal, its diet and environment.
Autochthonous	Indigenous to a particular habitat
Autogenic factors	Regulatory forces exerted by the microorganisms.
Congenic	Genetically identical except for a particular gene
Conjugative	Has the ability to conjugate. Transfer of genetic material by the process of conjugation.
CR	Colonisation resistance
CV	Conventional
Exogenous	Coming from the exterior, an invader.
GMMO	Genetically modified microorganism
Intra species	Between the same species
Inter species	Between different species
NIH guidelines	Guidelines for the work with recombinant DNA issued by the National Institute of Health. USA. Published in the Federal Register.
USEPA	United States Environmental Protection Agency (USA)
Wildtype	A strain isolated from nature

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Study of a Conditional Suicide System for Biological Containment of Bacteria in Germ-free Rats

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The effect of a biological containment system in *Escherichia coli* based on the stochastic induction of a lethal gene, *hok*,[8] was investigated in the gastrointestinal system of 33 germ-free rats. *E. coli* BJ16, identical to *E. coli* BJ4 originally isolated from a rat, but containing a plasmid pPKL100 with the *hok* gene, was given to germ-free rats. In these rats a plasmid-free and a plasmid-containing population was formed and co-existed in the gut. When given to gnotobiotic rats which had been initially monoassociated with *E. coli* BJ4, the *E. coli* BJ16 (hok^+) was eliminated at a faster rate than *E. coli* BJ17, identical to *E. coli* BJ4 but carrying a plasmid pMG33 without the *hok* gene. Expressed as T_{90}, the time used for a 90 per cent reduction of the bacterial concentration, the mean elimination for *E. coli* BJ16 (hok^+) was 2·8 d and for *E. coli* BJ17 (hok^-) was 5·3 d, indicating an effect of the *hok* gene in a competition situation.

KEY WORDS—Biological containment; *Escherichia coli*; Gnotobiotic rat; Conditional suicide.

INTRODUCTION

The concept of biological containment is an important element in the safe use of genetically modified microorganisms (GMMs). The ability of GMMs to survive and colonise is of considerable interest in the risk assessment related to human health. The classical containment systems consist of debilitated bacteria and non-conjugative, non-mobilisable plasmids. Such systems have limitations, especially in connection with deliberate release into the environment, where GMMs are supposed to survive and compete.

Molin and co-workers have developed a biological containment system based on the stochastic induction of a killing function.[8] It includes a plasmid (pPKL100) in which the toxin gene *hok* (host kill) is activated at random by an invertible promoter sequence, fimA. The *hok* gene codes for a small polypeptide (52 aa), lethal to a number of bacterial species. In competition with related bacteria, the killing function is expected to lead to the non-conditional elimination of the bacteria carrying the suicide plasmid.

This construction has been studied *in vitro*.[8] The aim of the present study was to investigate the fate of a wild type *Escherichia coli* containing the suicide plasmid in the intestine *in vivo*. The intestine is the natural habitat of *E. coli*, and it is also a likely route of human exposure in connection with the release of GMMs. The germ-free rat was chosen for this study in order to eliminate the influence of microbial interaction other than the interaction under scrutiny. The intestine of the germ-free rat provides a complex biological environment compared to the simple laboratory test-tube, yet it has the advantage of easy detection of added test bacteria in contrast to the situation in a conventional rat with a complex microbial gut flora. In the gnotobiotic rat it is possible to study the fate of transformed *E. coli* containing the *hok* gene alone (monoassociation) or in competition with the original, isogenic *E. coli* (diassociation).

The experimental design in our investigations was based upon a measurement of the effect of the *hok* gene upon the rate of decline of a secondary invader in the gut of the gnotobiotic rat.

MATERIALS AND METHODS

Animals

A total of 33 (16 female and 17 male) germ-free Han:Wist rats, (4–10 mth old at the start of the experiment) were kept in flexible isolators (Isotec type 12134). The rats were given 50 kGy-irradiated Altromin 1314 and 50 kGy-irradiated drinking water

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Table 1. *E. coli* dosing concentrations in five groups of germ-free rats

Group	No. of rats at EOE	Dose at T1 (c.f.u.)	T1–T2 (d)	Dose at T2 (c.f.u.)	T2-EOE (d)
I	5	$2\cdot6\times10^9$ BJ4	22	$4\cdot0\times10^8$ BJ16	125
II	5	$2\cdot6\times10^9$ BJ4	22	$4\cdot6\times10^7$ BJ16*	125
III	5	$2\cdot6\times10^9$ BJ4	33	$4\cdot4\times10^7$ BJ17	50
IV	6			$2\cdot5\times10^8$ BJ16	74
V	5			$6\cdot0\times10^8$ BJ4	47

T1 = time at first dosing; T2 = time at last dosing; EOE = end of experiment.
*Anaerobically cultured BJ16.

ad libitum. All animals were housed individually in type III macrolon cages with 50 kGy-irradiated hardwood bedding (Red special, Spanwell). All animals were acclimatised to the test environment (minimum 1 wk) and the germ-free status of the rats was checked before the start of the experiment. In order to check for the presence of bacteria other than the test bacteria, faecal samples from all rats were seeded at least once during the experiment on PCA (Oxoid CM 463).

The flexible isolators had a negative pressure during the experiment in order to avoid unintentional release of GMMs. Air inlets as well as air exhausts were supplied with Hepa filters (Interfilta limited, grade IFO). The ambient room temperature was maintained at approximately 20°C with a light period from 9.00 p.m. to 9.00 a.m. Relative humidity was approximately 55 per cent. Air circulation 8–10 times per hour.

Bacteria and plasmids

An *E. coli* strain originally isolated from a Wistar rat (Mol:Wist) was tested and found sensitive to a broad range of antibiotics including ampicillin and tetracycline. This strain, designated BJ4, has been characterised by F. and I. Ørskov at Statens Seruminstitut, Copenhagen, Denmark as an *E. coli* rough:K^-:H2 strain. Plasmid constructions were kindly supplied by the Molin group (Technical University of Denmark). Plasmid pPKL100 is a pBR322 derivative carrying the fimA promoter, the *hok* gene and expressing ampicillin resistance.[9] pMG33 (identical to pOU1033) is a pBR322 derivative without the fimA promoter and the *hok* gene and expressing tetracycline resistance.[11] Each plasmid was transformed into *E. coli* BJ4, resulting in BJ4(pPKL100) designated BJ16 and BJ4(pMG33) designated BJ17. Transformation was done according to Maniatis.[10] Bacterial cultures used for dosing were grown in Luria-Bertani broth overnight at 37°C either semi-aerobically, i.e. static cultures in capped tubes, or strictly anaerobically, i.e. static cultures in capped tubes in a Wise anaerobic working station (Don Whitley Scientific Ltd). The stationary phase cultures were centrifuged at 5000 ***g*** for 5 min and resuspended in physiological saline with 0·1 per cent peptone (Oxoid).

Experimental procedure

Thirty-three rats divided in five groups were given 1 ml bacterial culture p.o. by gavage according to Table 1. Two rats (from group I and III) died shortly after initial dosing and *E. coli* BJ4 was isolated from spleen, liver and intestinal lymph nodes. In the time period T1 to T2 faecal samples were taken regularly. Two rats from group I, two rats from group II and one rat from group III were sacrificed interim in order to examine the intestinal distribution of *E. coli* BJ4.

Faecal samples were taken directly by provoked defaecation in connection with manipulation of the animals. As the act of provoked defecation is not always successful, the use of this procedure meant that a variable fraction of the animals (10–20%) at any given date could not be sampled. This drawback was accepted in order to ensure fresh samples. The faecal samples were processed in the laboratory immediately.

Bacteriological quantification

The samples of faeces and intestinal contents were diluted in 10-fold series in physiological saline with 0·1 per cent peptone (Oxoid). The bacterial concentration was estimated using the spread plate

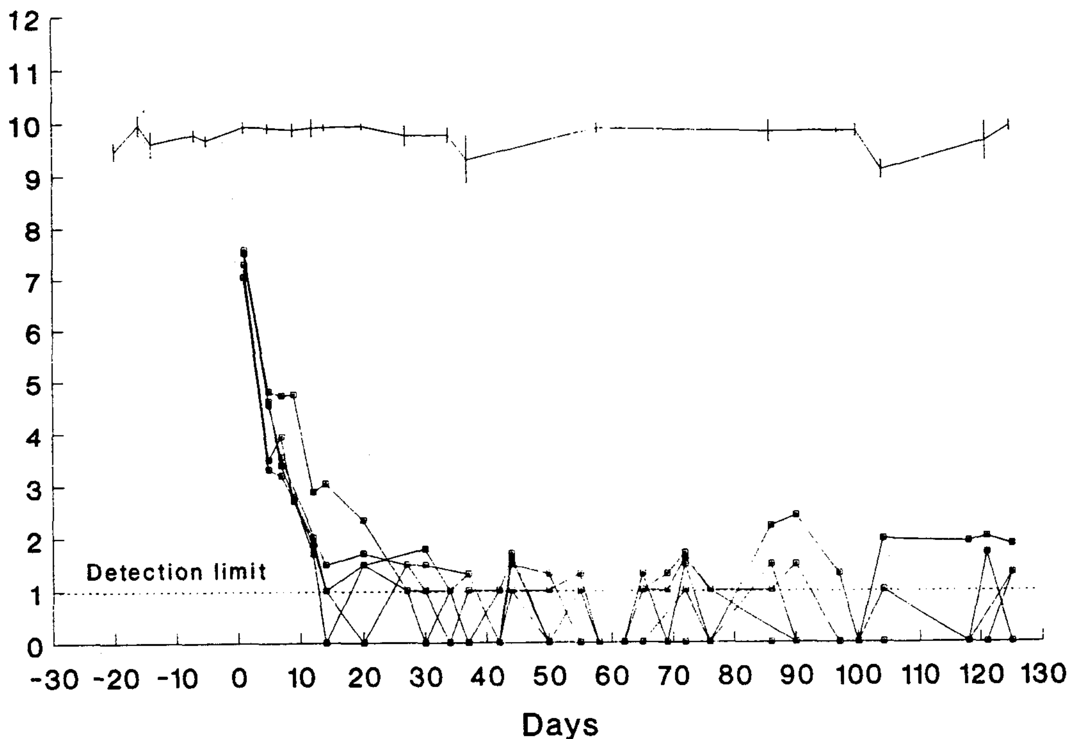

Figure 1. *E. coli* concentrations in faecal samples from group 1 of gnotobiotic rats. Five germ-free rats were initially associated with *E. coli* BJ4 and secondly at day 0 associated with semi-aerobically cultured *E. coli* BJ16 (*hok*⁺, Ap). BJ16 is a transformed *E. coli* BJ4 carrying a plasmid pPKL100. *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with ampicillin. The mean total *E. coli* concentration (—) from the five rats is shown. Vertical lines represent standard deviations. The concentration of ampicillin-resistant *E. coli* (□) from each of the five rats is shown

method. The general detection limit was 10 c.f.u./g faeces. The incubation was at 37°C for 24 h on MacConkey agar (Bacto MacConkey Agar, Difco) and MacConkey agar supplemented with 50 µg/ml ampicillin (Sigma A9518) and 12·5 µg/ml tetracycline (Sigma T3383), respectively.

Statistical analysis

Bacterial counts were transformed to $\log_{10}$ values and linear regression analysis was done using PROC GLM procedure of SAS (release 6·03, SAS Institute, Inc).

RESULTS

Results from the analysis of faeces are presented in Figures 1–4. Figure 1 presents the results from group I. Five rats were monoassociated with BJ4 and later given semi-aerobically cultured BJ16. Figure 2 shows the results from group II where five rats were monoassociated with BJ4 and later given anaerobically cultured BJ16. In both groups the total concentration of *E. coli* in faeces was high, approximately 10^9–10^{10} c.f.u./g faeces and remained relatively constant at this level throughout the experimental period. After diassociation at day 0 with BJ16 the concentration of ampicillin-resistant *E. coli* declined for 10–20 days from approximately 10^7 c.f.u./g faeces at day 1 to less than 10^2 c.f.u./g faeces, and persisted at this low level throughout the experimental period. The total *E. coli* counts in the period of diassociation covered BJ4 as well as BJ16, but since BJ16 never constituted more than 1 per cent of the total count the BJ4 concentration can reasonably be equivalated to the total *E. coli* count.

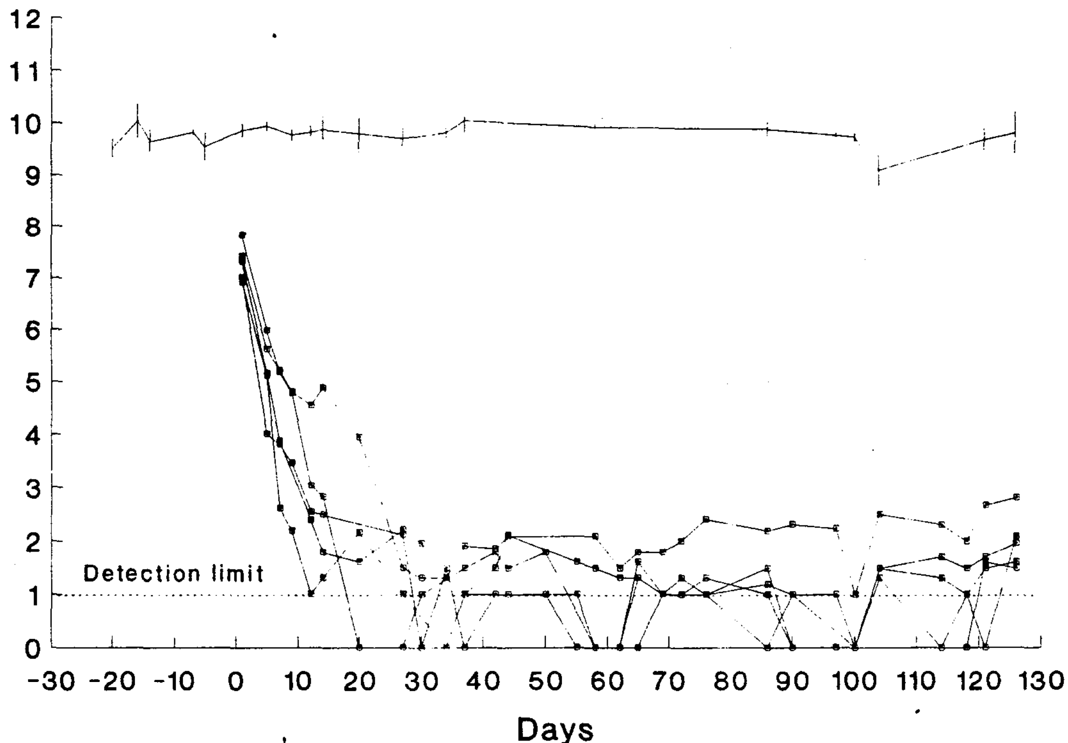

Figure 2. *E. coli* concentrations in faecal samples from group II of gnotobiotic rats. Five germ-free rats were initially associated with *E. coli* BJ4 and secondly at day 0 associated with anaerobically cultured *E. coli* BJ16 (*hok*$^+$, Ap). BJ16 is a transformed *E. coli* BJ4 carrying a plasmid pPKL100. *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with ampicillin. The mean total *E. coli* concentration (—) from the five rats is shown. Vertical lines represent standard deviations. The concentration of ampicillin-resistant *E. coli* (□) from each of the five rats is shown

Figure 3 presents the results from group III. The five rats were monoassociated with BJ4, and later given semi-aerobically cultured BJ17. After diassociation at day 0 the concentration of tetracyclin-resistant *E. coli* declined below the limit of detection (10 c.f.u./g faeces) within 15–40 d.

The concentration of BJ16 and BJ17 seemed to decline exponentially. The data were therefore analysed in linear regression analyses ($\log_{10}$ of bacterial concentration versus time) and the results of these are shown in Table 2. Using a Student *t*-test the *P* values below 0·05 signify a regression line with a slope significantly different from 0. The slope values from Table 2 were used to calculate T_{90} values for each animal. T_{90} represents the time period used for a reduction in bacterial concentration of 90 per cent (one $\log_{10}$ unit). T_{90} values were then used to compare the elimination rates of test bacteria in the three groups using a Student *t*-test approach (Table 3). No significant difference could be found between the mean T_{90} values in groups I and II, whereas the mean T_{90} of groups I + II was significantly lower than the mean T_{90} of group III.

Figure 4 shows the results from the animals in group IV which were given BJ16 only. It was seen that initially the total *E. coli* concentration in the faeces was close to the ampicillin-resistant *E. coli* concentration (approximately 10^9 c.f.u./g faeces). At day 8 all animals showed a total *E. coli* concentration around 10^{10} c.f.u./g faeces and an ampicillin-resistant *E. coli* concentration close to 10^8 c.f.u./g faeces. The total *E. coli* concentration remained at 10^{10} c.f.u./g faeces in all six rats, and in five of these the ampicillin-resistant *E. coli* concentration seemed to stabilise 2–4 $\log_{10}$ units below

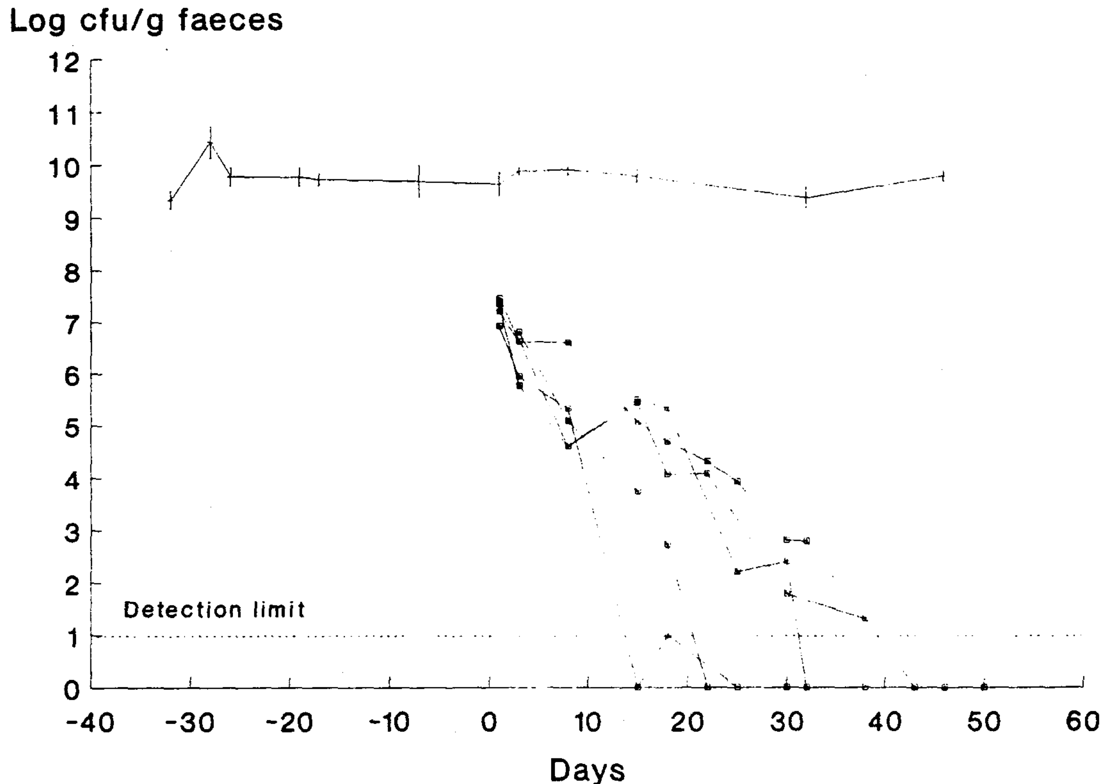

Figure 3. *E. coli* concentrations in faecal samples from group III of gnotobiotic rats. Five germ-free rats were initially associated with BJ4 and secondly at day 0 with *E. coli* BJ17 (*hok*⁻, Tc). BJ17 is a transformed *E. coli* BJ4 carrying a plasmid pMG33. *E. coli* counts were made on MacConkey agar (total) and on MacConkey agar supplemented with tetracycline. The mean total *E. coli* (—) from the five rats is shown. Vertical lines represent standard deviations. The concentration of tetracycline-resistant *E. coli* (□) from each of the five rats is shown

this. Only in one rat the ampicillin-resistant *E. coli* concentration increased following the initial decline, to reach a final concentration almost identical to the total *E. coli* concentration.

In group V the five rats were given BJ4 alone. The *E. coli* concentration was 10^9–10^{10} c.f.u./g faeces. During the 50 d sampling period ampicillin-resistant *E. coli* were only detected in three samples out of a total of 30 samples and tetracyclin-resistant *E. coli* were detected in two of a total of 27 samples with a detection limit of 10 c.f.u./g faeces. In all five positive samples the concentration of antibiotic-resistant *E. coli* was below 10^2/g faeces.

Figure 5 shows the gastrointestinal distribution of BJ4 in five monoassociated rats, 12 d after dosing. BJ4 was present in high numbers in all parts of the gastrointestinal system, but especially abundant in caecum, colon and faeces. Analysis of variance showed no significant difference between the mean concentrations in caecum, colon and faeces.

DISCUSSION

The monoassociation of the germ-free rats with *E. coli* BJ4 resulted in a uniform colonisation level in the gut in the order of 10^9–10^{10} c.f.u./g faeces of this microorganism (Figures 1–3). Possible sex- and age-related differences affecting the results were not evaluated, because of the great similarity among individuals. Results from interim sacrifice showed a growing concentration of *E. coli* throughout the gut, starting with a relatively high concentration in the ventricle (10^4–10^6 c.f.u./g) (Figure 5). The most likely explanation for these high concentrations is the lack of a normal microflora in germ-free rats. It should be noted that there was no significant difference between the mean concentrations of *E. coli* BJ4 in the caeca colons and faeces of the five rats. The faecal concentration could therefore be said to reflect the *E. coli* concentrations in the caecum and colon.

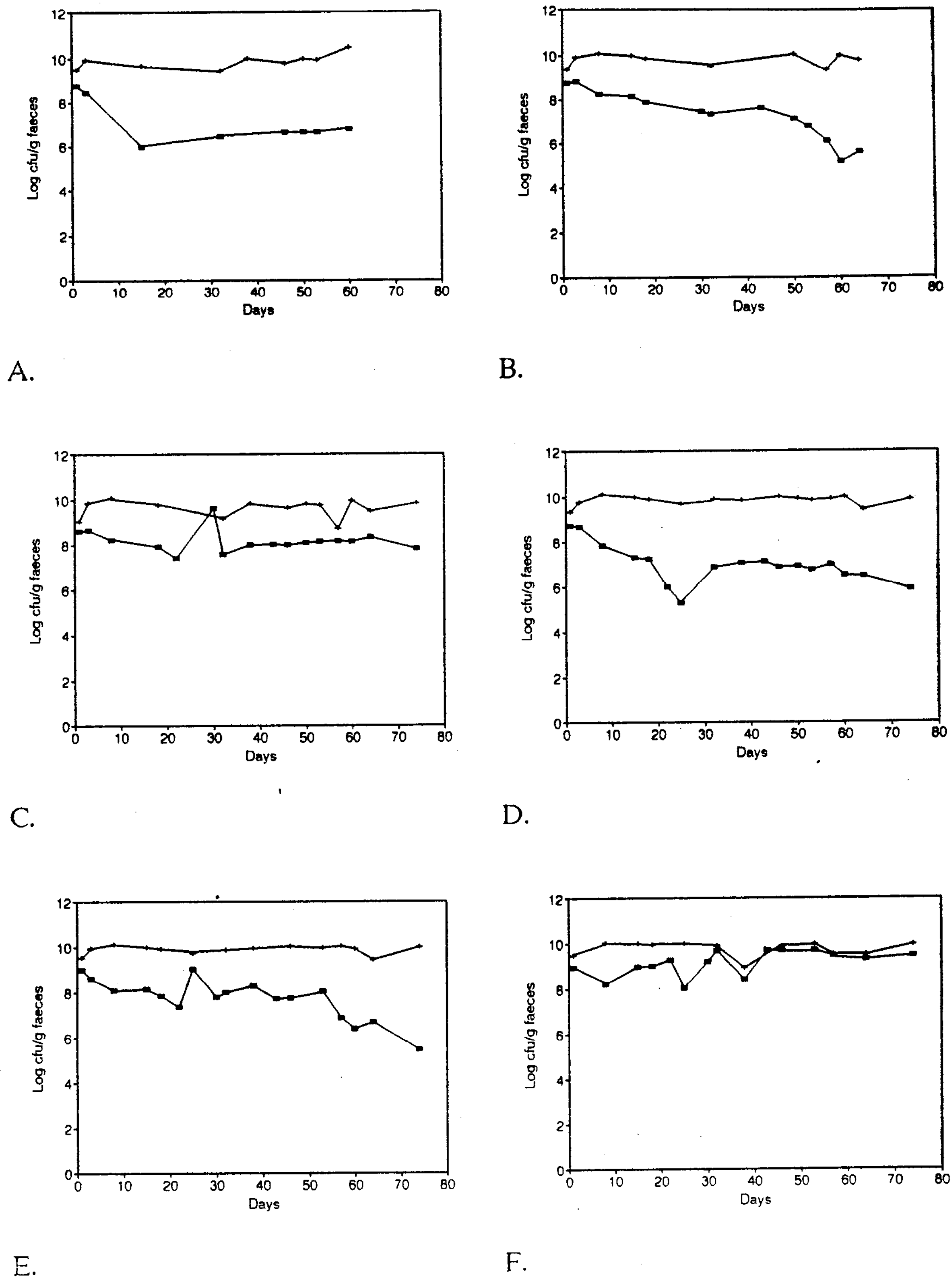

Figure 4. *E. coli* in faecal samples from rats 1–6 (A–F) in group IV is shown. The germ-free rats were monoassociated with *E. coli* BJ16 (*hok*$^+$, Ap). BJ16 is a transformed *E. coli* BJ4 carrying a plasmid pPKL100. Total counts of *E. coli* (+) were made on MacConkey agar and counts of plasmid containing *E. coli* BJ16 (■) on MacConkey agar supplemented with ampicillin

Table 2. Slope coefficient (α) for the decrease in faecal concentration of *E. coli* BJ16, *hok*$^+$ Ap (group I and II) and *E. coli* BJ17, *hok*$^-$ Tc (group III) in 15 gnotobiotic rats previously colonised with *E. coli* BJ4

Group	Animal no.	Samples (no.)	Slope coefficient (α)	SE	$P(>t)$
I	3	6	−0·43	0·074	0·0042
I	4	3	−0·33	0·039	0·0743
I	7	6	−0·34	0·060	0·0051
I	8	6	−0·47	0·071	0·0027
I	9	6	−0·48	0·050	0·0007
II	1	6	−0·32	0·065	0·0076
II	3	6	−0·39	0·025	0·0001
II	4	6	−0·47	0·079	0·0042
II	5	5	−0·42	0·040	0·0019
II	6	6	−0·18	0·016	0·0001
III	3	7	−0·15	0·037	0·0105
III	5	4	−0·26	0·023	0·0075
III	7	7	−0·16	0·025	0·0014
III	8	10	−0·16	0·007	0·0001
III	9	4	−0·34	0·044	0·0167

SE = standard error of α.
$P(>t)$ = probability for a higher t value under the 0 hypothesis: $\alpha = 0$; i.e. when this value is lower than 0·05, α is significantly different from 0.

Table 3. Comparison (t test) of mean T_{90} values for the decrease in faecal concentration of *E. coli* BJ16, *hok*$^+$ Ap (group I and II) and *E. coli* BJ17, *hok*$^-$ Tc (group III) in 15 germ-free rats previously colonised with *E. coli* BJ4

Group	n	Plasmid	T_{90} (d)	SEM	$P(>t)$
I+II	10	*hok*$^+$	2·8	0·34	0·0048**
III	5	*hok*$^-$	5·3	0·78	

T_{90} = the time used for 90% reduction in bacterial concentration.
SEM = standard error of the mean.
**Statistically significant difference using a t test.

Two rats died 2 d after dosing. At autopsy *E. coli* BJ4 was found in intestinal lymph nodes and in the liver and spleen in low concentrations. Physical damage through dosing as well as direct translocation should be considered as possible explanations. Translocation of non-pathogenic microorganisms from the gut has been reported previously in connection with bacterial overgrowth of *E. coli* in gnotobiotic mice.[1,12] However, no further cases of translocation have been observed at our laboratory during at least 68 dosings of BJ4 or derivatives of BJ4 to germ-free rats subsequent to this experiment (results not shown). It is therefore most likely that the findings of *E. coli* in the tissue of the rats in this experiment were caused by physical damage through the dosing procedure.

When a second *E. coli* strain was given to the monoassociated rats the newly introduced strain rapidly declined. This exclusion of a second invader of intestinal niches has been reported previously.[2] The concept of exclusion of invading microorganisms through pre-occupation of niches is also considered to be at least part of the explanation behind the colonisation resistance of the conventional intestinal microflora.[4,11] The experimental design in our investigations was based upon a measurement of any effect of the *hok* gene upon the rate of decline of a secondary invader in the gnotobiotic rat. Therefore it was imperative to obtain a situation with competition between two closely related strains

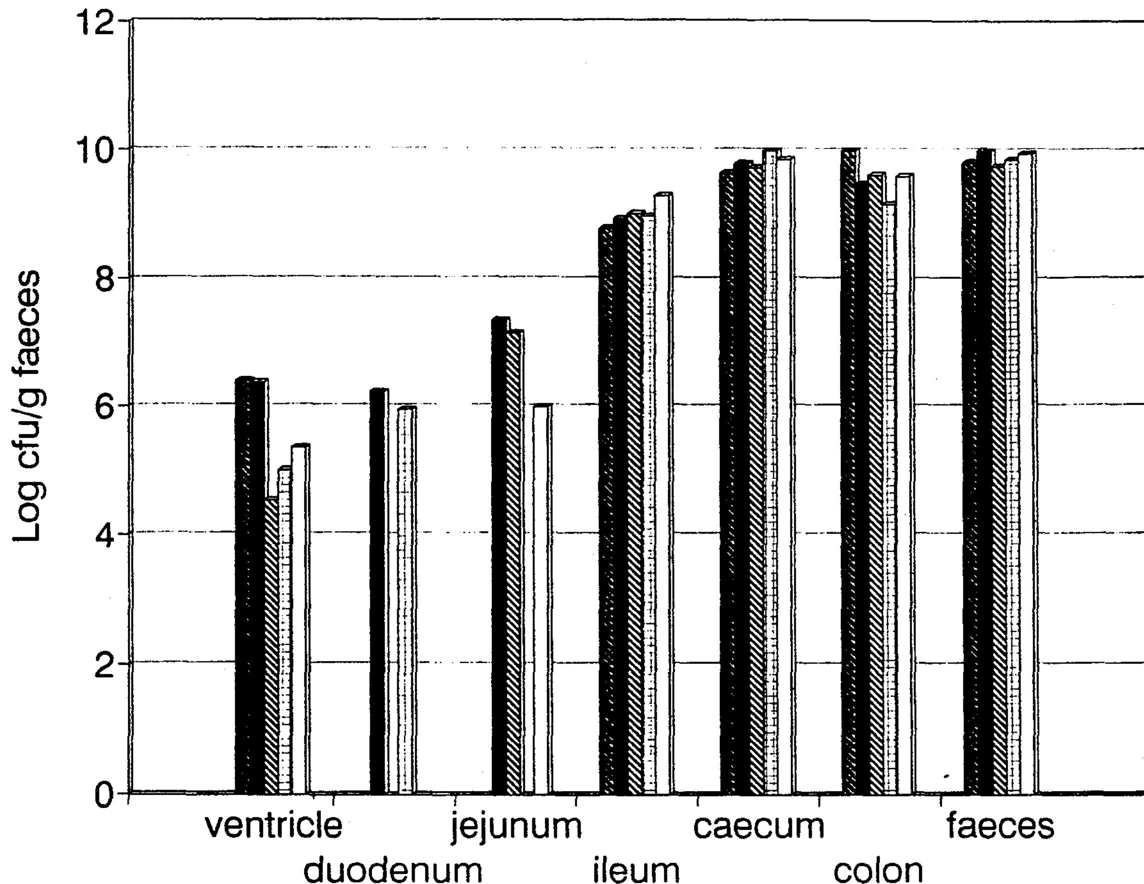

Figure 5. Distribution of *E. coli* BJ4 in the gastrointestinal system of five monoassociated rats. Each bar represents the concentration of BJ4 in the gastrointestinal contents of one rat. Bacterial concentration of the intestinal contents in duodenum and jejunum were only obtained for two and three of the rats respectively

leading to the possible exclusion of the second strain. This is a situation which is most likely to occur in nature. The concentration of the second *E. coli* strain declined exponentially, whereas the total *E. coli* level was constant. The results from the linear regression analyses of the log transformed data were in agreement with log linear elimination of this secondly added *E. coli* in groups I–III (Table 2).

The fact that the mean T_{90} values of groups I and II were not statistically significantly different showed that the elimination rate of the second strain did not depend upon semi-aerobic/anaerobic cultivation before dosing (Table 3). It should be noted that the dosing concentration was higher in group I than in group II (Table 1). The differences in dosing concentrations are not considered to interfere with the interpretation, as we have generally found in other trials that the dosing concentration has no influence upon the subsequent concentration in the gnotobiotic gut when the dose given is higher than 10^4 c.f.u.

The combined mean T_{90} of BJ16 (*hok*$^+$) in group I and II was significantly lower than the mean T_{90} of BJ17 (*hok*$^-$) in group III (Table 3), when using ampicillin resistance as the indirect marker for the presence of the *hok* gene; this meant that the strain carrying the plasmid with the *hok* gene was eliminated at a higher rate than the strain carrying a similar plasmid without *hok*. The obvious explanation could be that the *hok* gene, as expected, conveys a disadvantage to the strain because a fraction of the population is constantly dying as a result of the stochastic induction of the *hok* gene.

Following the decline period, in groups I and II the *E. coli* BJ16 *hok*$^+$ seemed to stabilise at a concentration around 10 c.f.u./g faeces (Figure 1), which could signify mutations in the *hok* gene. Mutations in a related suicide system have been

reported to take place *in vitro* at a rate of 10^{-6} to 10^{-7} per generation time.[6] Another less plausible explanation could be that at this point in time the growth rate of BJ16 (*hok*$^{+}$) exceeded the death rate caused by the stochastic induction of the *hok* gene. This would correspond to a lag phase of 15–20 days, which however does not correspond to the absence of a lag phase in group IV, where BJ16 (*hok*$^{+}$) was monoassociated to the rats (Figure 4). A third explanation could be the spontaneous formation of ampicillin-resistant *E. coli* BJ4. This, however, does not correspond to the results in group V, where the rats were only dosed with *E. coli* BJ4 and where such spontaneously formed ampicillin-resistant colonies were only rarely found.

The *E. coli* BJ17 (*hok*$^{-}$) did not stabilise and was eliminated totally from the rats in group III (Figure 2). It should be noted, however, that these rats were only followed for 30–40 days after the *E. coli* BJ17 (*hok*$^{-}$) had reached the detection limit, whereas the rats in groups I and II were sampled for 100 days after the *E. coli* BJ16 (*hok*$^{+}$) reached the detection limit (10 c.f.u./g faeces). The criterion to terminate the rats was three consecutive samplings with a result below the detection limit. This criterion was only met by the rats in group III. Working from the assumption that a mutation in the *hok* gene did occur in the BJ16 (*hok*$^{+}$) strain, the reason why a similar mutation did not show up in the BJ17 (*hok*$^{-}$) could be that the 'control' plasmid without *hok* conveyed no disadvantage to the *E. coli* strain.[3] The reason for the total elimination of the BJ17 (*hok*$^{-}$) population could then be that the expression of the marker gene in this group (tetracycline resistance) resulted in a lowered fitness of this population. It has been reported[7] that the expression of a tetracycline marker gene could have an adverse effect on the reproductive fitness of plasmid-containing (pBR322-derivative) *E. coli.*

In the control group IV where BJ16 (*hok*$^{+}$) was dosed alone, there was no elimination of the bacterial population. The results clearly show that under the optimal growth conditions in the gnotobiotic rat, the growth rate exceeds the stochastic death rate.

In the same group a subpopulation of *E. coli* without the plasmid was formed in all rats (Figure 4). However, in no animal was a decline comparable to groups I, II or III observed. In all six rats the two subpopulations co-existed throughout the experimental period of 74 days. In rat nos 1–5 the plasmid-containing population constituted 0·01–1 per cent of the total count. In rat no. 6 the plasmid-containing population constituted 10–50 per cent of the total count. These differences could be incidental, relating to the formation in time and space of the two different populations in the physical niches of the gut.

It is difficult to differentiate between the contribution of loss of plasmid and the effect of the *hok* gene upon the elimination of the ampicillin-resistant *E. coli* population. However, it might be reasonably assumed that the loss of plasmid (and even the biological variation in the mode of loss) could be the same for the plasmid-containing groups I–IV, whether or not the plasmid pBR322 coded for the *hok* gene. Following this reasoning it could be argued that the difference in estimated T_{90} values, 5·27 d of BJ17 and 2·84 d of BJ16, was a true effect of the *hok* gene.

The results still leave a number of parameters to be directly estimated. In order to elucidate further the effect of this kind of biological containment system, supplementary investigations including plasmid constructions with identical antibiotic markers as well as chromosomally marked *E. coli* are under preparation.

ACKNOWLEDGEMENT

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THE USE OF GERM-FREE RATS FOR THE STUDY OF FATE AND EFFECT OF GENETICALLY MODIFIED MICROORGANISMS.

Bodil Jacobsen, Jørgen Schlundt, and Grethe Fischer

SUMMARY:

Using germ-free rats and *Escherichia coli* as testbacteria we have studied two plasmidconstructions made for biological containment of genetically modified bacteria. Both constructions are based on the principle of random activation of a gene, *hok* or *gef*, belonging to the same family of killing genes and coding for proteins lethal to *E.coli*. The effect of the killing genes is reflected in reduced decimation time, T_{90} values, of *E.coli* in a situation of competition. The preliminary results of this study shows an effect of the *gef* gene in accordance to with the effect of the *hok* gene previously obtained.

INTRODUCTION

Developments in biotechnology have put focus on genetically modified microorganisms (GMM's) in relation to human health. Important aspects in the risk assessment of GMM's is the ability of a GMM to survive and multiply as well as the possibility of transfer of genetic material to other microorganisms in a natural environment.
The lack of a normal microflora in the germ-free rat permits colonisation in high concentrations of testbacteria. This makes it possible to study biological interactions, which would be difficult to study in a conventional rat. In germ-free rats and with *Escherichia coli* as testbacteria we have studied two different genetic constructions made for high biological containment. Both constructions are based on the principle of activation of an inserted gene coding for a protein lethal to *E.coli* (Molin et al. 1987, Molin et al.,1993). Data obtained from investigations with a plasmid construction based on activation of the gene *hok*, indicated an effect of this gene in form of a reduced decimation time of *E.coli* carrying the *hok* gene in a situation of competition (Jacobsen et al.,1993). This investigation raised a number of questions. Therefore we have continued these investigations.
In the present study, from which we present some of the preliminary results, we used identical antibiotic markers for all plasmid constructions and the testbacteria containing the suicide plasmid could be identified by a chromosomal marker. The lethal gene used in these construction was *gef* (Poulsen et al.,1991), a gene very similar to *hok*, belonging to the same family of killer genes (Gerdes et al.,1990). The activation of the *gef* gene was accomplished by the use of a an invertible promoter sequence of the *fim*A gene, which can switch from a "OFF" to an "ON" configuration, the socalled switch. The constructions also included the regulatory genes *fim*B and *fim*E. The *fim*B gene product mediates an "ON" configuration and *fim*E gene product an "OFF" configuration of the *fim*A promoter (Klemm 1986, McMaclain et al.,1991). Similar to the investigation of the effect of the *hok*

gene, the experimental design was based upon a measurement of the suicide gene upon the decimation time of a secondary invader in the gut of the gnotobiotic rat.

METHODS

Animals

14 germfree Han:Wist rats (approximately 10 weeks old) were kept individually caged in flexible isolators. The isolators had a negative pressure during the experiment, in order to avoid unintentional release of the GMMs. Air-inlets as well as air-exhaust were supplied with HEPA-filters.

Bacteria and plasmids

Escherichia coli BJ4 (Jacobsen et al., 1990, Jacobsen et al.,1993), sensitive to a broad range of antibiotics including nalidixin and kanamycin , *E.coli* BJ19, a selected nalidixin resistant mutant of BJ4 (Nielsen and Schlundt, 1992). Plasmidconstructions were kindly supplied by S. Molin, The Technical University of Denmark. pSM910 contains switch-*gef*, *fim*B and *fim* E, producing the *fim*B gene product in surplus. pSM1020 contains only the switch (S. Molin, personal communication) Both plasmids are Km^r and were separately transformed into BJ19. A solution of spores from *Bacillus stearothermophilus* was used as a marker for the intestinal transit rate (Ducluzeau et al., 1970)).

Experimental procedure

In group A (7 rats) each rat was given p.o 1 ml ($4.0x10^8$ c.f.u.)BJ4 and 18 days later 1 ml($6.2x10^8$ c.f.u.) BJ19:pSM910 together with a sporesuspension of approximately $3x10^8$ c.f.u. *B.stearothermophilus* . In group B (7 rats) each rat was given p.o 1 ml ($4.7x10^8$ c.f.u.) and 13 days later 1ml ($9.6x10^7$ c.f.u.) BJ19:pSM1020 together with a sporesuspension of $3x10^8$ c.f.u. *B.stearothermophilus*. The two groups were kept in separate isolators.

Bacteriological quantification

Serial dilutions of faecal samples taken from the rectum was made. The bacterial concentrations of *E.coli* was estimated using the spread plate method, using MacConkey agar (Oxoid CM115) and the relevant antibiotics in the following concentrations: Kanamycin (Sigma K1377) 50µg/ml, nalidixin (Sigma N4382) 40µg/ml. *B. stearothermophilus* was estimated according to Ducluzeau et al.,1970.

RESULTS

After diassociation the total *E.coli* counts in both groups remained high, between 10^9 and 10^{10} c.f.u/g faeces throughout the experimental period. The populations of *E.coli* BJ19:pSM910 and BJ19:pSM1020 as well as *B. sterarothermophilus* declined rapidly and finally the kanamycin resistant *E.coli* and Bacillus spores were eliminated from both groups of rats.
In order to further elucidate and compare *E.coli*, with and without the *gef* gene, regression analysis and estimates of T_{90} were made. When a linear

regression line can be fitted to the data, T_{90} is a meaningful measure of decline. T_{90} (decimation time) being the time used for 90% reduction in bacterial concentration. The calculations of the decimation time T_{90} of BJ19:pSM910 and BJ19:pSM1020 is shown in table 1.

DISCUSSION

In our previous study of the effect of the *hok* gene on bacterial decimation times, the results indicated an effect of this gene, i.e. a reduced decimation time of *E.coli* in a situation of competition. When given to gnotobiotic rats which had been initially monoassociated with *E.coli* BJ4, the *E.coli* containing a plasmid with the *hok* gene decreased at a faster rate than *E.coli* containing a plasmid without the *hok* gene. Expressed as T_{90} the mean reduction time for *E.coli* (*hok*$^{+}$) was 2.8 days and 5.3 days for *E.coli* (*hok*$^{-}$) In the experimental design we used different antibiotic resistances as markers for the presence (ampicillin) or absence (tetracycline) of the *hok* gene. We concluded that expression of the marker (resistance to tetracycline) could not be eliminated as having an effect on the decimation time of the *hok*$^{-}$ plasmidcontaining populations of *E.coli*(Jacobsen et al.,1993).
In this study we used identical markers (resistance to kanamycin) in the plasmidconstructions and the difference between the constructions were the presence or absence of the *gef*, *fim*B and *fim*E genes. The use of resistance to nalidixin as a marker for the *E.coli* host BJ19 also allowed for direct estimation of plasmid loss (results not shown). When comparing the two groups of rats diassociated with *E.coli* BJ4 and *E.coli* BJ19:pSM910 and BJ4 and BJ19:pSM1020, we find a significant difference in mean T_{90} values (Table 1). The *E.coli* containing the *gef* gene has a mean T_{90} value of 2.5 days in contrast to 6.6 days of *E.coli* without the *gef* gene. This in accordance with our previous results obtained with the *hok* gene.
In relation to the use of an intestinal transit marker we observed (data not shown) that *E.coli* with the *gef* gene was eliminated from faecal samples before the marker and that *E.coli* without the *gef* gene was eliminated slower or at the same rate as the intestinal marker. This would correspond to an intestinal population of *E.coli*(*gef*$^{+}$) which died during the intestinal passage, whereas the population of *E.coli*(*gef*$^{-}$) was still dividing during the passage. This also strongly supports the conclusion that the *E.coli* are killed by the induction of the *gef* gene.

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Table 1: Comparison (t-test) of mean T_{90} values for the decrease in faecal concentration of *E.coli* BJ19:pSM910 (*gef*$^{+}$) (group A) and *E.coli* BJ19:pSM1020 (*gef*$^{-}$)(group B) in 14 germ-free rats previously colonized with *E.coli* BJ4.

Group	n	Plasmid	T_{90} (days)	SEM	P (>t)
A	7	gef^{+}	2.5	0.5	0.0017**
B	7	gef^{-}	6.6	0.9	

T_{90}= the time used for 90 per cent reduction in bacterial concentration.
SEM= standard error of the mean.
** Statistically significant difference using a t test.
P(>t)= probability for a higher t value under the hypothesis: T_{90} values are equal.

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Development and Testing of Improved Suicide Functions for Biological Containment of Bacteria

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We have developed very efficient suicide functions for biological containment based on the lethal *Escherichia coli relF* gene. The suicide functions are placed in duplicate within a plasmid and arranged to prevent inactivation by deletion, recombination, and insertional inactivation. The efficiency of this concept was tested in a plasmid containment system that prevents transfer of plasmids to wild-type bacteria. Protection against plasmid transfer was assayed in test tubes and in rat intestine. Protection was efficient and refractory to inactivation by mutation and transposons. The efficiency of the suicide system was also tested in soil and seawater. We show that unprecedented suicide efficiency can be achieved in soil and seawater after suicide induction by IPTG (isopropyl-β-D-thiogalactopyranoside). More than 7 orders of magnitude reduction in suicide bacteria was achieved.

As genetically engineered bacteria gain more widespread use in bioremediation, agriculture, and medical industry, built-in containment systems become more attractive. If the environmental impact of introduced or escaping bacteria can be minimized by suicide systems triggered by preprogrammed conditions, then the use of these bacteria becomes of less concern.

Descriptions of such suicide systems have been published previously (see reference 17 for a review). These systems have been based on lethal genes from *Escherichia coli* and have been triggered by tryptophan deficiency (18), plasmid transfer (11, 18), IPTG (isopropyl-β-D-thiogalactopyranoside) (2), and degradation of xenobiotic compounds (6). However, the deficiency of all these suicide systems is their inefficiency: a substantial fraction (often 10^{-4}) of bacteria is allowed to escape suicide even in model tests under optimal laboratory conditions. We have previously identified the factors limiting the efficiency of a *relF*-based suicide system to be mutational inactivation and selection of mutants (11). The mutation rate of a single suicide function was determined to be 10^{-6} per cell per generation. Leaky repression of suicide was demonstrated to lead to growth inhibition of suicide-positive populations, causing the selection of mutants that have lost suicide function. We described a two-plasmid system of duplicated suicide functions with a resulting reduction in mutation rates (11). This principle was applied to a suicide system in *Pseudomonas putida* in which two suicide systems were placed on the chromosome (9). This report describes the preparation and testing of a new single-plasmid suicide system that overcomes the problems of mutation and mutant selection. With efficiencies as high as those demonstrated, the concept of biological containment could gain more widespread use. To demonstrate the usefulness of this new system, we show its application to a plasmid containment system. Plasmid containment addresses the concern of transfer of genetically engineered traits to wild-type bacteria in the environment (21). The new plasmid containment system is tested both under controlled laboratory conditions and in a more complex environment, the rat intestine. We also test the new suicide functions in soil and seawater by inducing them with IPTG.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1. Plasmid pSK360 contains two *relF* suicide genes transcribed from the synthetic promoter $P_{A1\text{-}03\text{-}04}$ that are identical to previously described single-suicide functions (11). Plasmid pSK360 was constructed from (starting from bp 1 of pSK360): nucleotides (nt) 1 to 86 from pUHE21-2 (12), nucleotides AAT TCCC from pUC9 (1), nt 1075 to 1350 from the *relB* operon (1), nucleotides GCAGCCA from pUC9, nucleotides CAAG from *Hin*dIII linker, nucleotides CT from an *Xba*I linker, nt 1120 to 140 from pUHE21-2, nucleotides TGGCTGC from pUC9, nt 1350 to 1075 from *relB* operon, nucleotides GGGAATT from pUC9, nt 85 to 1 from pUHE21-2, nt 3414 to 3230 from pUHE21-2, nt 3832 to 1425 from pACYC184 (5), nucleotides AG from *Xba*I linker, nucleotides CTTG from *Hin*dIII linker, nt 3254 to 3848 from pBR322 (7), and nt 2903 to 3414 from plasmid pUHE21-2. A detailed description of plasmid pSK360, including DNA sequence and restriction sites, can be obtained from the authors upon request (or can be retrieved via Internet Gopher site dna.cedb.uwf.edu). The replication origin between the *Pvu*II sites of pSK360 was replaced by plasmid pBOE93 (a kanamycin-resistant RSF1010 derivative [18]), opened at the *Xmn*I site to yield the fusion plasmid pSK360::pBOE93. The control fusion with plasmid pBR322 was constructed with the *Eco*RI site of both plasmids to yield pBR322::pBOE93. BJ19 is a nalidixic acid-resistant derivative, selected on a nalidixic acid plate, of a wild-type *E. coli* isolated from rat feces.

Media. All media were NY rich media (11) unless noted otherwise. Solid media were NY medium with agar and antibiotics added (tetracycline, 20 mg/ml, and ampicillin, 100 mg/ml). IPTG (no. I5502; Sigma Chemical Co., St. Louis, Mo.) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma no. B4252) were added to concentrations of 1 mM and 40 mg/ml, respectively, where indicated. Filters used on top of solid media were nitrocellulose BA85.

Matings. Host strain BD3432 was mated on an NY plate as described previously (11) with either recipient CSH55 or the control recipient BD3434 with *lac* repressor.

Triparental matings. Donor strain BD3349 was grown to an optical density at 436 nm of 0.5 in NY medium, and 0.5 ml was mixed with 0.5 ml of recipient strain BD3347, harboring either pDW205, pSK360, or control plasmid pBR322 with the same optical density, and incubated without agitation for 1 h at the bottom of a 50-ml bottle at 37°C. A second recipient strain, XAC, was added (0.5 ml), and incubation was continued for 1 h. Ten milliliters of NY medium was then added, and incubation was continued for 2 h with agitation. Aliquots were plated on NY medium plus nalidixic acid plus kanamycin and NY medium plus nalidixic

TABLE 1. Bacterial strains and plasmids

E. coli strains or plasmid	Genetic markers	Origin	Reference
E. coli strains			
HB101	(*coliB*) *hsdS20*(r_B^-,m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Str^r) *xyl-5 mtl-1 supE44*		3
MC1000	*araD139* Δ(*ara leu*)7697 Δ*lacX74 galU galK strA*		4
BD3364	HB101/F′ *lacI*^{q1} *lacZ*::Tn*5* (*kan*)		11
S17.1	*recA1 pro thi hsdR hsdM*$^+$ (RP4)		20
BD3432	S17.1/pR2172 + pBOE::pSK360		This work
BD3379	S17.1/pBOE::pBR322		This work
BD3346	MC1000 *lacI*^{q1}		11
BD3347	MC1000 *lacI*^{q1} *recA1 srl*::Tn*10*(*tet*)		11
CSH50	Δ(*lac-pro*) *ara strA thi*		15
CSH55	Δ(*lac-pro*) *supE nalA thi*		15
BD3434	CSH55/pR2172		This work
BD3349	CSH50/F′ *lacI*::Tn*5*(*kan*) *lacZ*$^+$		This work
XAC	Δ(*lac-pro*) *argE*(Am) *nalA rif*		16
BJ19	Nal^r derivative of rat intestine isolate		This work
BD3392	BD3347/pSK360		This work
Plasmids			
pUHE21-2	*bla* $P_{A1-03/04}$	ColE1	12
pACYC184	*cat tet*	p15A	5
pSK360	*bla tet* 2 × ($P_{A1-03/04}$-*relF*)	ColE1	This work
pDW205	*bla lac* P_{UV5}-*relF*	ColE1	11
pR2172	*bla lacI*^{q1} (pBR322 *tet*::*lacI*^{q1})	ColE1	11
pBOE93	RSF1010 *kan*	IncQ	17
pBR322	*bla tet*	ColE1	7
pSK360::pBOE93	*bla tet kan*	IncQ	This work
pBR322::pBOE93	*bla tet kan*	IncQ	This work

acid plus ampicillin plus tetracycline, counting plasmid transfer rate as the number of transferred plasmids per transferred F′.

Enhanced triparental mating. The enhanced triparental mating experiment was performed as the triparental mating experiment described above except for the following. Transconjugants from the first mating were enriched on NY medium plus kanamycin plus tetracycline overnight before mixing with the second recipient. This modification increased the rate of control plasmid transfer, thereby lowering the detection limit. The transfer rate of the suicide plasmid was below the detection limit, so the detection limit is given.

Fluctuation experiment (14). Twenty-two cultures of approximately 1,000 cells of BD3364/pSK360 in 10 ml of NY medium were grown to 5×10^8 cells per culture. The cultures were harvested by centrifugation and plated on agar plates containing the inducer IPTG and the chromogenic β-galactosidase substrate X-Gal as described previously (11).

Suicide kinetics. BD3364/pSK360 was plated on nitrocellulose filters (BA85) on NY plates containing IPTG. At intervals, filters were transferred to plates without IPTG for incubation.

Cointegrate transfer. Donor strain BD3432, harboring cointegrate pSK360::pBOE93, was mated on a plate as described previously (11) (0.2 ml each of donor and recipient was mixed on NY plates and incubated for 12 h before resuspending and plating dilutions on selective plates) with either recipient strain CSH55 or BD3434 (*lacI*^{q1}).

Plasmid transfer in the rat intestine. To allow sufficiently high concentrations of donor and secondary host strains to detect transfer, germ-free (Han:Wist; Zentralinstitut für Versuchtierzucht, Hannover, Germany) rats were caged individually in isolators and fed KGy-irradiated Altromin 1314 (Brogaarden Gentofte, Denmark) and water ad libitum. They were fed donor strain BD3432 (1 ml of 10^8 cells per ml) by gavage three times over 7 days. The density of donor bacteria in the feces reached 10^8 CFU per g of feces. Then, on day 12, a recipient (BJ19) was added, and transconjugants were monitored in the feces for 7 days. Feces were sampled from the rectum, and dilutions were plated on selective plates. As a control, the plasmid fusion pBR322::pBOE93 in S17.1 was added on day 7 in control experiments. Plasmid transfer rates are averages of five experiments.

Seawater experiments. Seawater was collected from Santa Rosa Sound (estuary, 2.6% NaCl) in the vicinity of Pensacola Beach, Fla., and filter sterilized (0.22-μm-pore-size cellulose acetate; Corning Corp., Corning, N.Y.). BD3392 cells were grown in Luria broth (LB), inoculated at less than 10^4 cells, harvested at the exponential phase, washed in seawater, and resuspended in seawater to about 10^7 cells per ml. The seawater was incubated in separate tubes (1 ml in capped autoclaved Pyrex 9820 tubes [18 by 150 mm]; Corning) at 30°C without shaking. To half of the tubes, IPTG was added to a final concentration of 0.5 mM. To half of the tubes with and without IPTG, glucose was added to a final concentration of 2% (wt/vol). Samples were taken periodically (two tubes were sacrificed for each sampling), diluted in 0.9% NaCl, and plated on LB plus ampicillin (100 μg/ml; Sigma) plus tetracycline (20 μg/ml; Sigma) plates (LAT plates; for total CFU determination). Low colony counts were determined either by plating 50 ml directly from the seawater on LAT plates or by filtering one to five tubes, each containing 1 ml, and layering the filter (0.2-μm-pore-size, 47-mm polycarbonate membrane filters; Poretics Corp., Livermore, Calif.) on LAT plates. The dilution factor obtained by plating directly from seawater containing IPTG (a 500-fold dilution) was sufficient to enable growth of IPTG-sensitive cells.

Samples were also plated on the same type of plates supplemented with 1 mM IPTG (LATI plates) to score for suicide-minus mutants.

Experiments in nonsterile seawater were performed in the same way, except that the seawater was not filter sterilized and samples were not taken in duplicate.

In experiments to test suicide in large cultures (see Fig. 7), 10 ml of seawater was inoculated with cells from outgrown cultures (stationary phase, >10^9 cells). These seawater tubes were incubated at room temperature and sampled once each day.

Soil experiments. One gram of potting soil (Hyponex Corp., Marysville, Ohio; sieved through a 2-mm-pore-size aluminum mesh) was added to glass culture tubes with caps (same as those described above) and sterilized by autoclaving. BD3392 cells were grown in LB medium plus antibiotics as described above, harvested in the exponential phase, and resuspended in sterile 0.9% NaCl. Then, 0.3 ml of cells in saline solution was added to each tube, and the tubes were incubated at 30°C. To some of the tubes, IPTG and/or glucose was added, together with the cells, to estimated final concentrations in soils of 2 mM and 2% (vol/vol), respectively. The added liquid made the soil moist without any liquid phase. CFU counts were determined by adding 3 ml of sterile 0.9% NaCl to a tube (two tubes were sacrificed for each sampling), vortexing for 60 s, letting settle for 60 s, and diluting in 0.9% NaCl. Dilutions were plated on LAT for total CFU and on LATI for enumerating suicide-minus mutants.

Statistical treatment of data. All sampling for Fig. 5 and 6 was performed in duplicate, sacrificing two tubes for each datum point. The mean value and standard deviation of these two numbers were used in the figures. When one sampling yielded colonies and the duplicate did not, the observed CFU and the detection limit were used for the mean and standard deviation calculations.

For practical reasons, IPTG was mixed with resuspended bacteria and glucose on ice and then immediately added to the seawater or soil experiments. The first sample was taken as quickly as possible (within 5 min), and yet the CFU counts in the tubes with IPTG were slightly lower than the CFU counts in the tubes with no IPTG. These results indicate that suicide had already begun before the first sample was taken, probably in the tubes while the sample was being extracted. The figures show the same CFU counts at time zero for experiments with and without IPTG because all experiments were inoculated from the same culture. The plating of samples directly from cultures containing IPTG diluted the IPTG 500 times and did not prevent growth of IPTG-sensitive cells. This was determined by comparing the colony counts on plates with direct plating with the colony counts on plates where the sample had been diluted 100 times prior to dilution (i.e., diluting the IPTG 100 times). In all experiments reported, the two numbers were comparable when corrected for the dilution factor, showing that the IPTG concentration had no effect on colony counts in this concentration range.

RESULTS

Preparation of a suicide plasmid with duplicated lethal system. To reduce the inactivation rate of the suicide function, a suicide gene was duplicated within a plasmid to yield plasmid pSK360 (Fig. 1). The suicide function is based on the lethal *relF* gene from *E. coli* (8). The function of this gene remains elusive, but it rapidly kills the cell when expressed from a foreign promoter. We used the synthetic promoter $P_{A1-03/04}$ (12), which is efficiently repressed by the *lac* repressor. The two copies of this suicide function were arranged in such a way that no single mutational event (deletion in particular) can lead to inactivation of both. A deletion including *ori* would lead to loss of plasmid replication, and a deletion including the *bla* and *tet* genes would lead to loss of selective markers (as well as any gene that is to be contained, which should be cloned in one of these markers). As depicted in Fig. 2, suicide can be induced within the host cell by adding the gratuitous inducer of the *lac* operon, IPTG. After such an IPTG induction, the fraction of cells surviving suicide can be counted by plating on media in the absence and presence of IPTG. To obtain a statistical

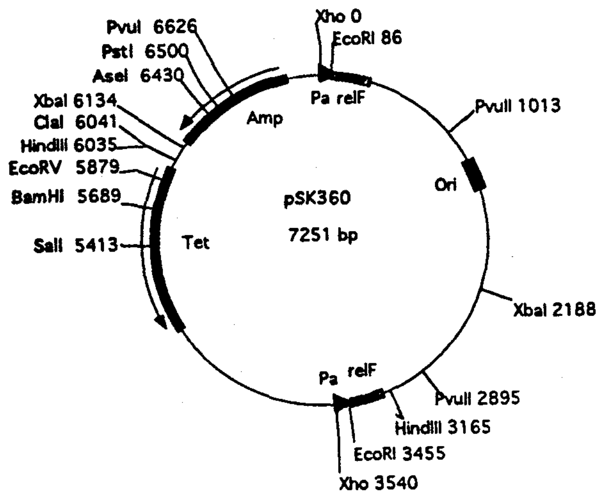

FIG. 1. Plasmid pSK360 containing duplicated killing functions. Unique sites suitable for cloning are indicated in boldface letters.

measure of the survival rate, a Luria-Delbrück fluctuation test was performed (11, 14): several IPTG plates were counted in parallel to quantify the zero fraction of the Poisson distribution describing the chance of mutation.

A fluctuation test was performed with BD3364/pSK360 as described in Materials and Methods. The host strain BD3364 contained the high-yield *lac* repressor gene *lacI*^{q1}, which is necessary for suicide repression, on an F' plasmid. The mutation rate to IPTG resistance was calculated to be 10^{-8} per cell per generation (mutants were scored on two of nine plates, with 10^8 cells plated on each plate). However, the presence of the chromogenic substrate X-Gal in the plates allowed us to distinguish colonies that survived because of inactivated suicide functions (the chromosomal *lacZ* gene allowed X-Gal cleavage and, hence, a blue phenotype after IPTG induction) (Fig. 2) from colonies with superrepressor mutations in the *lacI* gene, rendering the repressor insensitive to IPTG (resulting in a white phenotype since the chromosomal *lacZ* could not be induced). Repeated experiments showed only white colonies (i.e., superrepressor mutants) and never any blue colonies. From the absence of blue colonies on all plates (21 plates) and the number of cells plated (5×10^8 per plate), the inactivation rate of both suicide functions is estimated to be less than 10^{-10} per cell per generation. This number is relevant for applications that do not rely on IPTG-induced suicide, for example, suicide after plasmid transfer.

The fluctuation tests described above were performed in a *recA* host. To determine whether homologous recombination between the suicide functions catalyzed by RecA has any effect, the fluctuation test was repeated in a *recA*$^+$ strain (BD3346). In this experiment, blue colonies occurred at a rate of approximately 10^{-8} per cell per generation, indicating that both suicide functions were inactivated at this rate. The homologous recombination, which the *recA*$^+$ strain allows, should lead only to inversion of the origin of replication. The increased frequency of loss of both suicide functions could be explained by mutation of one killer gene and then by loss of the other via gene conversion in connection with the recombination. One caveat is that the *recA*$^+$ and *recA* strains used for comparison are not otherwise isogenic. Thus, the difference in inactivation rate cannot be attributed conclusively to the RecA protein.

Suicide kinetics. The fluctuation experiments described above, in which suicide is defined as the absence of colonies on

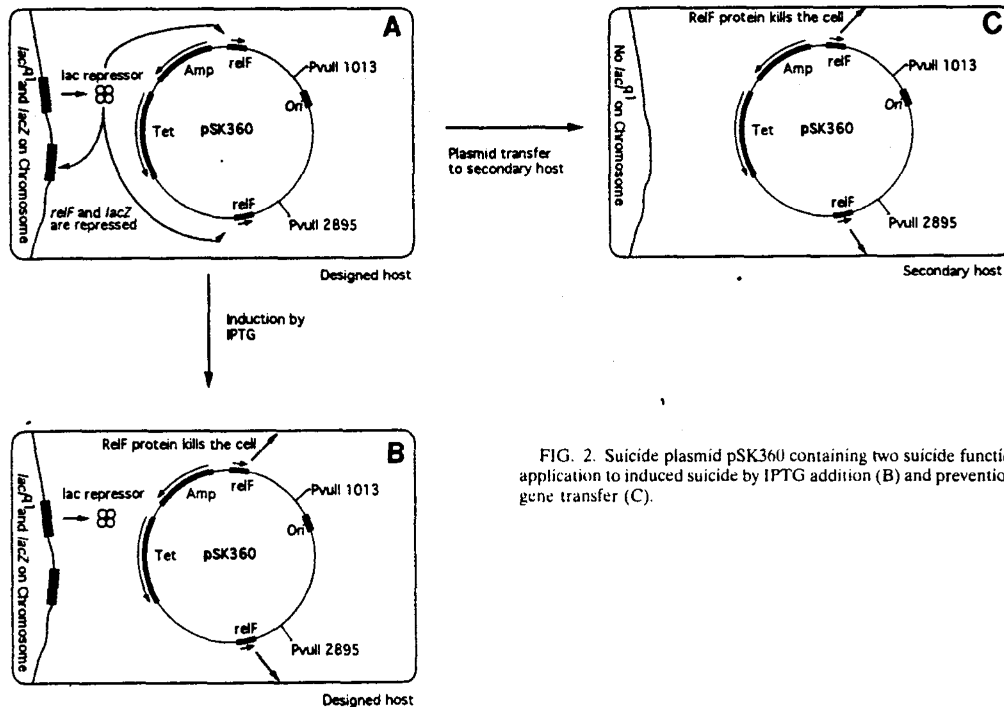

FIG. 2. Suicide plasmid pSK360 containing two suicide functions (A) and its application to induced suicide by IPTG addition (B) and prevention of horizontal gene transfer (C).

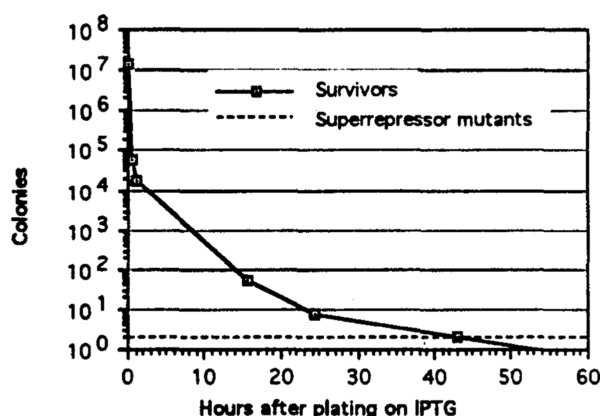

FIG. 3. Suicide kinetics after IPTG induction of strain BD3392, which carries plasmid pSK360. After different incubation times on IPTG plates, cells on filters were moved to plates without IPTG, and colonies formed after incubation were counted.

plates containing IPTG, do not provide any information on the kinetics of suicide nor do they distinguish between bacteriostatic and bacteriocidal effects. To gain insight into the exact kinetics of the suicide process, BD3392 cells were plated on filters placed on plates containing IPTG. At different times, these filters were transferred to plates without IPTG to identify cells that were still able to form colonies (Fig. 3). The results confirm that the suicide function is bacteriocidal but that it takes 40 to 50 h before the most persistent fraction of 10^{-8} cells disappears. Superrepressor mutants form colonies on IPTG plates but remain constant in number throughout the experiment and can be distinguished from nonmutated colonies that do not form until after they are transferred to plates without IPTG.

Application to plasmid containment. The suicide plasmid pSK360, as described above, is derived from the plasmid pBR322. It is not self-transmissible, but there are several avenues of transfer to secondary hosts. Such transfer may be of concern if the plasmid carries cloned genes that can be transferred to secondary hosts in the environment. Plasmid pBR322 can be transferred to secondary hosts by transduction, transformation, or mobilization by conjugative plasmids (11, 13). Suicide functions on the plasmid can prevent or reduce the spread of recombinant DNA by killing transconjugants following transfer when suicide functions are derepressed in secondary hosts that lack *lacI*^{q1} (Fig. 2).

Prevention of cointegrate-mediated plasmid transfer. (i) Nutrient agar. In a triparental mating experiment, an F′ plasmid carrying two transposons entered the cell harboring suicide plasmid pSK360 or control plasmid pBR322. Transfer of pSK360 or pBR322 (by transposon-mediated cointegrate formation with F′ [10]) to the recipient was monitored by selecting for ampicillin and tetracycline resistance. Transfer of pBR322 (measured as the number of transferred plasmids per transferred F′ plasmid) was readily detectable (1×10^{-4}), whereas transfer of suicide plasmid pSK360 was below the detection limit ($<4 \times 10^{-7}$). For comparison, the single suicide function in plasmid pDW205 offered little protection against plasmid transfer (transfer rate, 10^{-5}), probably because the chance of transposon integration into the suicide function was very high (about 1 in 10 if random insertion were assumed).

To determine if any limit in the protection against plasmid transfer could be detected, the potential for transfer was enhanced by studying the transfer of a premade cointegrate between pSK360 and a self-transmissible RSF1010 derivative. This fusion yielded a conjugative suicide plasmid. Although the transfer rate of the pSK360::pBOE93 to the recipient with the *lacI*^{q1} repressor (control; BD3434) was very high (10^{-1} transconjugants per donor [BD3432]), transfer to the recipient that did not contain the repressor (CSH55) was below the detection limit ($<10^{-7}$ transconjugants per donor).

(ii) Rat intestine. The efficiency of the plasmid containment was tested in the rat intestine to demonstrate the efficiency of the suicide function in preventing the spread of recombinant DNA in vivo. The *E. coli* host has its normal habitat in mammalian intestines. Thus, we chose to test our plasmid containment in the rat intestine. However, to allow colonization of the intestine by the debilitated laboratory strains which we were using, germfree rats without detectable microflora had to be used. Figure 4A shows the mean results of five rat experiments using the control plasmid pBR322::pBOE93. The host with the control plasmid colonized the intestine (as monitored by colony counts on diluted rat feces); the recipient (nalidixic acid resistant) was then administered. Transconjugants were detected at a level of 10^2 to 10^3 CFU/g of feces from the day after administration of the secondary host to the end of the experiment. However, when the same experiment was performed with the contained plasmid (Fig. 4B) (mean of five experiments), no transconjugants were detected, although the same concentration of host and secondary host as that in the control experiment was used.

It should be noted, however, that the donor with the contained plasmid appears to compete more poorly with the recipient than the donor with the control plasmid. To compensate for differences in donor concentrations between control experiment and contained experiment, transconjugants per donor were calculated. Pooling all samples after addition of recipient BJ19, a total of 3.4×10^3 donors with the control plasmid and a total of 7.5×10^7 transconjugants with the control plasmid were detected. Thus, the ratio of transconjugants to donors in the control experiment was 4.6×10^{-5}. In the experiment with contained plasmid, no transconjugants were detected (detection limit, 1), but a total of 1.0×10^8 donors was detected. Thus, the ratio of transconjugants to donors was less than 1.0×10^{-8} or at least 550 times less than that in the control experiment.

Suicide induction in soil and seawater. Initial experiments examined the efficiency of suicide in sterile soil. Figure 5 shows the effect of IPTG on the viability in soil of strain BD3392, which carries pSK360. Without IPTG, CFU counts remained nearly stable for the 28 h of the experiment. With IPTG, CFU counts dropped below the detection limit after 28 h (the last datum point is below the detection limit, which is equal to 0.2 CFU/g of soil; see Discussion). The decline in CFU after the addition of IPTG is more than 7 orders of magnitude. No significant difference was detected in the presence of glucose. Samples were also plated on LATI plates to detect suicide-minus mutants. No mutants were detected during the experiment (a detection limit of 66 CFU/g of soil).

The effect of IPTG on the viability of strain BD3392 in sterile seawater is shown in Fig. 6. Again, CFU counts dropped dramatically in the presence of IPTG, although the rate of suicide was lower than that observed in soil. Adding glucose and IPTG led to an increased rate of suicide. The reduction in CFU was more than 6 orders of magnitude after the addition of IPTG. No suicide-minus mutants were selected on LATI plates during the experiment (a detection limit of 24 CFU/ml).

In all of the experiments presented above, no suicide-minus

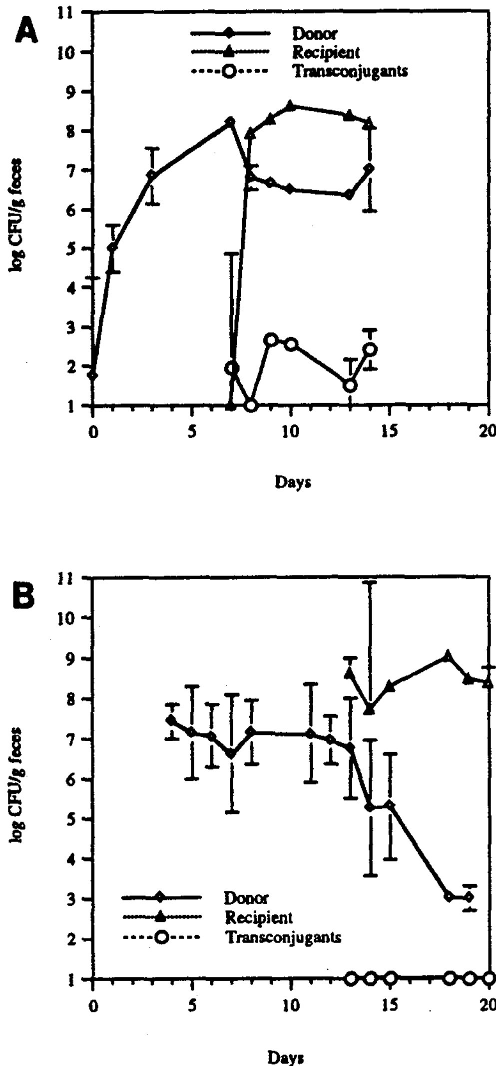

FIG. 4. Plasmid transfer in rat intestine. (A) Donor contains control plasmid pBR322:pBOE93; recipient is *E. coli* BJ19 (average of five rat experiments); (B) donor contains containment plasmid pSK360::pBOE93 (average of five rat experiments). The detection limit was 1 log(CFU) in all experiments. Datum points on the *x* axis [1 log(CFU)] indicate determinations that were below the detection limit.

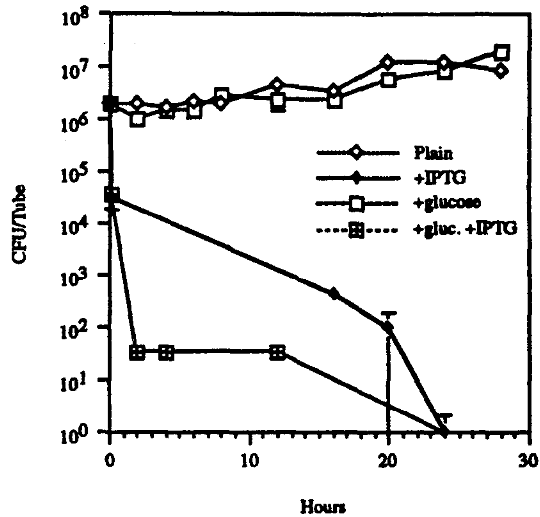

FIG. 5. Effect of IPTG and glucose on viability in sterile soil of strain BD3392, which carries plasmid pSK360. Samples labeled plain were taken from soil with no amendments. Viability was determined as CFU on LAT plates. Vertical bars indicate standard deviations from duplicate experiments. The detection limit was 0.2 CFU per tube. Datum points on the *x* axis indicate determinations that were below the detection limit.

mutants were detected. To demonstrate how selection of such mutants would have affected the population dynamics in sterile seawater, an overnight culture large enough to contain spontaneous suicide-minus mutants (above 10^9 cells, a culture is likely to contain mutants that arise at a rate of 10^{-8} per cell per generation) was used for inoculating the seawater. These mutants were monitored by plating on LATI, enabling growth of only suicide-minus mutants. After an initial decline in total CFU (detected by plating on LAT) and a rapid growth of mutants, mutants constitute the majority of the about 10^4 cells in the tube after 6 days (Fig. 7). In the absence of IPTG, the mutant population followed the trend of the total population of cells: after an initial small increase, mutant counts dropped below the detection limit 3 days after the inoculation of the tubes.

Figure 8 shows the decline in CFU in nonsterile seawater with and without glucose. Without glucose, the decline in CFU occurs at about the same rate with and without IPTG, presumably reflecting competition and predation from indigenous species. In the presence of glucose, the introduced cells are stabilized for a short time. The addition of IPTG leads to rapid suicide.

DISCUSSION

We have shown in this report how an efficient suicide plasmid can be designed by duplication of a suicide function within

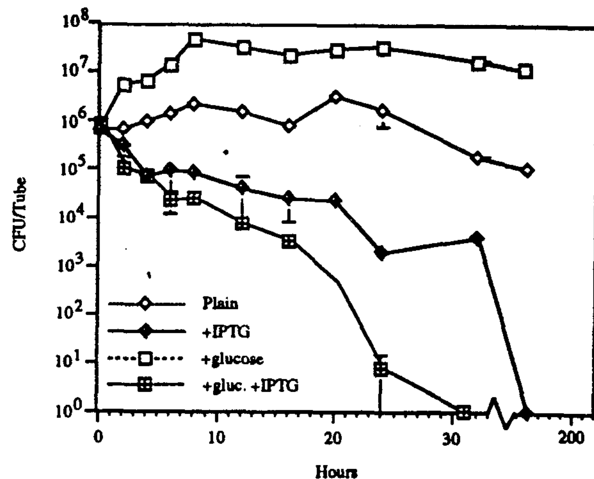

FIG. 6. Effect of IPTG and glucose on viability of strain BD3392 in sterile seawater. Viability was determined as CFU on LAT plates. Vertical bars indicate standard deviations from duplicate experiments. The detection limit was 0.2 CFU per tube. Datum points on the *x* axis indicate determinations that were below the detection limit. Note that the *x* axis is broken between 30 and 200 h.

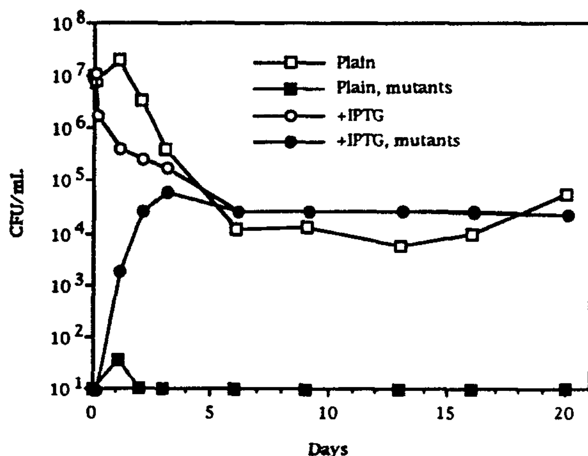

FIG. 7. Effect of IPTG on viability of strain BD3392 in sterile seawater. Spontaneous suicide-minus mutants were present in the inoculum. Viability was determined as CFU on LAT plates. Mutants (filled squares and circles) were detected as CFU on LATI plates. The detection limit was 5 CFU/ml. Datum points on the x axis indicate determinations that were below the detection limit.

a plasmid. Furthermore, we have shown how two suicide functions provide protection against inactivation by transposons (compare cointegrate-mediated transfer rates of pDW205 and pSK360) and that transfer of a plasmid from its host strain to a secondary host can be efficiently prevented (diminished by at least a factor of 10^6) under laboratory conditions. As one example of environmental conditions very different from optimal growth conditions in the laboratory, we tested transfer in rat intestine. Also in this situation, transfer was reduced below the detection limit (at least 550 times below the transfer rate of the control plasmid). Thus, we propose that the plasmid pSK360 can be used as a general cloning vector with reduced

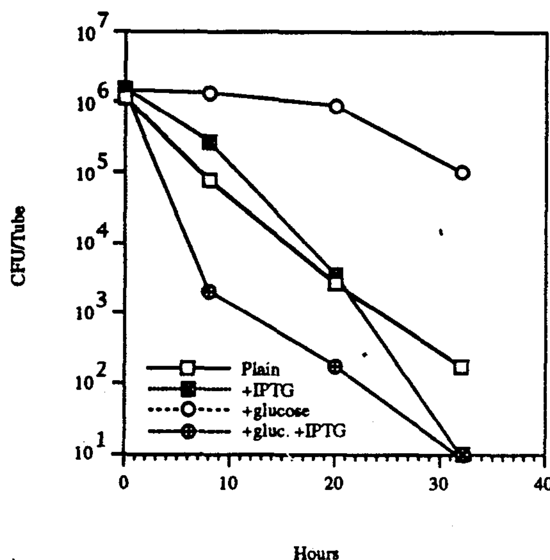

FIG. 8. Effect of IPTG and glucose on the viability of strain BD3392 in nonsterile seawater. Viability was determined as CFU on LAT plates. The detection limit was 20 CFU per tube. Datum points on the x axis indicate determinations that were below the detection limit.

transfer potential wherever risk concerns warrant it. While no system can provide 100% protection, an efficient system as we describe it can be a significant element in any risk evaluation. The plasmid would not be useful as a cloning vector if the suicide functions disturbed the cell during normal handling. We have previously shown that a single, well-repressed suicide function has no measurable effect on exponential growth in the laboratory (11). The suicide functions have no measurable effect on exponential growth in the laboratory (11). The suicide functions should also be useful for cloning on the chromosome, to protect against the transfer of genes cloned on chromosomes.

The concept of basing containment of a given cloned gene on the expression of a lethal gene has the advantage that expression of the cloned gene in a foreign host bacterium is likely to result in expression of the lethal gene as well. In secondary hosts where the lethal gene is not expressed, the cloned gene is unlikely to be expressed and is then of less concern. Even in a foreign host recognizing *E. coli* promoters but containing the wild-type *lacI* gene, suicide results because of inadequate repression in the presence of moderate numbers of repressor molecules (11).

If the target of the containment is a host and not a plasmid, the suicide functions should be placed on the chromosome of the host. Such a system has been demonstrated by Jensen et al. (9). We have tested the efficiency of such host containment by IPTG induction of our plasmid-based dual suicide system inside its host. By analyzing the reasons for survival in complex environments such as soil and seawater, we have been able to achieve much more efficient suicide than that previously reported.

We interpret our results as follows. If suicide-minus mutants were present in the inoculum of the sterile seawater, IPTG-induced suicide of wild-type cells would eliminate competition and cause rapid proliferation of these mutants (Fig. 7). In seawater without IPTG, suicide-minus mutants had no apparent growth advantage (Fig. 7). In all other experiments (Fig. 5, 6, and 8), mutants were not detected, and all wild-type cells containing the suicide plasmid committed suicide. Thus, the efficiency of suicide (i.e., the fraction of survivors after suicide induction) appears to be limited only by the mutation rate. If the inoculum is prepared without mutants (see Materials and Methods), then all inoculated cells commit suicide. That limits the inoculum to about 1/(10× mutation rate) cells, or about 10^7 cells in our case, where the mutation rate is about 10^{-8} per cell per generation.

Whereas the suicide efficiency is dependent only on mutation rate, the suicide rate (i.e., the kinetics with which the suicide-induced cells die) depends on other factors. One such factor is nutrient availability, as demonstrated by the addition of glucose to seawater (Fig. 6).

In soil experiments, we cannot exclude the possibility that cells adhering to soil particles and resisting extraction survive. The recovery from soil immediately after inoculation is 85% (data not shown), but we have not determined the extraction efficiency after 1 week of incubation. In seawater we have no such problems, because the contents of an entire tube can be plated.

This interpretation of our results explains previously published results in sterile soil microcosms. Recorbet et al. (19) reported that the reduction in CFU after sucrose addition to *E. coli* cells with a sucrose-induced suicide gene, *sacB*, is approximately 3 orders of magnitude. They also reported that suicide-minus mutants constituted a fraction of 10^{-5}, which might have arisen at a rate of 10^{-6} per cell per generation, a rate which we have previously found for a single suicide function of a com-

parable gene size (11). Thus, mutants were highly likely to be present in an inoculum of 5×10^6 per microcosm, and their results show a selection of these mutants from an undetermined (but low) number to approximately 5×10^4 mutants per microcosm after induction of suicide.

Similarly, a paper by Bej et al. (2) reported a reduction of 2 orders of magnitude in CFU after induction of a single *lacP-hok* suicide function with IPTG.

This selection of mutants, however, may not occur in nonsterile microcosms or in the environment because of competition and predation. Indeed, Recorbet et al. reported a rapid elimination of suicide survivors from nonsterile microcosms.

Thus, the limit in efficiency of suicide is determined largely by the mutation rate of the suicide system. Under sterile conditions, suicide is poor if the inoculum is large enough to contain mutants. Under nonsterile conditions and, perhaps, in the environment, the die-off of the introduced microbes by predation and competition may reduce the population size, and proportionally the number of suicide mutants, to levels that will prevent survival following induction of the suicide system.

In this report, we have used two measures to describe suicide efficiency: the difference in CFU counts before and after suicide induction and the difference in CFU counts between tubes with and without IPTG. To facilitate comparisons between different systems, we suggest using the former number to report suicide efficiency.

When contemplating bacterial containment by activation of suicide functions following field applications, a number of issues must be addressed. (i) Plasmid-borne suicide functions will contain (eliminate) plasmid-borne genes, including prevention of plasmid transfer (6), but will not contain the hosts that have lost the plasmid. If the host has to be contained, the suicide functions have to be moved to the chromosome. A chromosomal insertion of the *sacB*-based suicide system has been demonstrated successfully (19). (ii) Induction by IPTG, lactose, or sucrose, which work in small microcosms, may not be practical on a larger scale because of the quantities involved and the potential difficulty of reaching all released bacteria. One solution could be to link induction of suicide to the degradation of a xenobiotic, as suggested by Contreras et al. for the P_m(TOL)-*gef*-based system (6). (iii) *E. coli*, which we have used in our present studies, may not be the bacterium of choice if competition with indigenous soil bacteria is desired. (iv) Mutation rates described in this and previous reports have been determined during rapid exponential growth. If the prevailing mutation type is caused by errors in replication, such a mutation rate, expressed per generation, could be valid in the environment. Potential stress-induced mutations, however, could affect the prediction of the number of mutants. Mutation rates under stress would need to be evaluated. In a first attempt to obtain such data, we allowed the seawater experiment described in Fig. 7 to stand for 10 days after the last datum point was taken, for a total of 30 days. The total CFU counts remained around 10^4 during this period, but no suicide-minus mutants were detected. These results indicate that stress (starvation)-induced mutations in this bacterium under these conditions had not occurred.

The mutation rates of our dual suicide system are not the lowest obtainable. Based on calculations presented by Knudsen and Karlström (11), a single suicide system will have a mutation rate of about 10^{-6} per generation. A dual system with a common regulation, such as the one used here, will have a rate of about 10^{-8} or lower, depending on whether trans-dominant regulatory mutations occur. A dual suicide system with independent regulation systems (induced, for example, by lactose and sucrose), might have a rate approaching 10^{-12}. A triple suicide system, with independent regulation and independent targets, could have an even lower mutation rate. Although multiple suicide systems that are totally independent of each other remain to be tested, it seems plausible that very efficient suicide in the environment can be achieved.

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Conjugal Transfer of Plasmid DNA between *Lactococcus lactis* Strains and Distribution of Transconjugants in the Digestive Tract of Gnotobiotic Rats

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The conjugational transfer of the plasmid pAMβ1 between two strains of *Lactococcus lactis* subsp. *lactis* was measured in the intestinal tract of four conventional and eight gnotobiotic rats. In the conventional rats both donor and recipient strains were eliminated and transconjugants were not observed. Germfree rats were dosed orally with the recipient strain and the donor strain carrying pAMβ1. In faecal samples, transconjugants were detected within the first few days after dosing, and the transconjugants established a stable population at a level of 10^3–10^5 less than the recipient strain. In one group of animals the donor strain was eliminated from the intestine shortly after introduction, but still the transconjugants colonised the intestine. Samples from duodenum, jejunum, caecum and colon were cultured 2 or 5 wks after dosing with the recipient strain. The concentration of transconjugants was approximately 10^4 c.f.u./g throughout the intestine, whereas the concentration of recipients increased from 10^4–10^5 c.f.u./g in jejunum to 10^8–10^9 c.f.u./g in caecum and colon. The plasmid pAMβ1 seems to have conveyed to the recipient a competitive advantage in the small intestine but not in the large intestine.

KEY WORDS—*Lactococcus lactis*; Gnotobiotic rat; Conjugation; Intestinal tract.

INTRODUCTION

The use of genetically modified microorganisms (GMMs) in food is now a reality. Genetically modified baker's yeast has been approved for baking purposes in the UK. Other GMMs will be approved for food use within the near future. The risk assessment of GMMs in relation to food use incorporates a number of different endpoints, one of which will be the gene transfer potential of the GMM used. The current knowledge about *in situ* gene transfer is limited and most studies in this area relate to gene transfer in test-tube environments.

Since gene transfer between microorganisms is generally a low probability event, the most likely areas in which transfer could take place would be in niches with high microbial concentrations. For microorganisms relevant in food such niches in the human would primarily be in the gastrointestinal tract.

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The determination of the plasmid transfer rate constant *in vivo* is very difficult as the growth rate and spatial distribution of the donors, recipients and transconjugants throughout the intestine is not well understood. Several approaches to estimate plasmid transfer has been used previously ranging from transconjugant–recipient ratios[13,31] to parameter estimation using mass action kinetics[10,20,26] including segregation rate, growth rate and accessibility of the recipient. As we cannot at present measure the growth rate *in vivo* the transconjugant/recipient ratio is used both *in vivo* and *in vitro*. The ratio will be a combination of plasmid transfer and growth rate of the transconjugant.

Gene transfer in the gastrointestinal tract was originally studied phenotypically in relation to the transfer of drug resistance between genera of the family Enterobacteriaceae.[16,19] Later, Duval-Iflah *et al.*[6] confirmed R-plasmid transfer between such genera in the digestive tract of gnotobiotic mice associated with human faecal microbiota, and

recently Doucet-Populaire *et al.*[5] have shown transfer of plasmid-bound resistance factors from enterococci to *Escherichia coli* in the digestive tract of gnotobiotic mice.

In the early days of recombinant technology Anderson[1] initiated investigations using primarily *E. coli* K12 as donor in the human intestinal system. These experiments showed that plasmid transfer could take place from *E. coli* K12 to indigenous *E. coli*, but the transconjugants only reached very low concentrations and were quickly eliminated from the intestine. Later Freter *et al.*[10] used *E. coli* as a model organism for the general investigation of plasmid transfer in natural ecosystems, i.e. the intestine. The use of *E. coli* has been relevant in this context because *E. coli* was extensively used for experimental recombinant technology, and because most *E. coli* strains are natural inhabitants of the mammalian intestinal system. However, *E. coli* is not relevant in the food application of GMMs.

A microorganism that could be relevant as a GMM in food is *Lactococcus lactis*, which is used worldwide in the production of cheese and other fermented milk products. Therefore *L. lactis* is a likely candidate as a donor of recombinant genetic material in the intestinal system, and since a number of different *L. lactis* strains gain access to the system through the normal diet it will also be a likely recipient in this environment. *L. lactis* is traditionally not considered to have a natural habitat in the human gastrointestinal tract.[29] However, in one of the largest investigations of the normal indigenous intestinal microbiota, Finegold *et al.*[8] isolated *L. lactis* in faecal samples from approximately 30 per cent of 141 humans investigated, and the mean faecal concentrations in the different diet groups varied between 10^6 and 10^9 c.f.u./g faeces. Plasmid transfer from *L. lactis* to *Enterococcus faecalis* has been shown in the intestinal tract of gnotobiotic mice, but here the transconjugant population was eliminated within 10 days after dosing.[14]

In this study we have measured the transfer rate of a conjugative plasmid, pAMβ1, between two *L. lactis* subsp. *lactis* strains in the gastrointestinal tract of germfree and conventional rats. The plasmid pAMβ1 has previously been used for *in vivo* plasmid transfer studies between *Lactobacillus reuteri* and *E. faecalis* in gnotobiotic mice where plasmid transfer was detected.[22] The germfree rats were included in the experiment because it has been shown that the colonisation resistance of the normal intestinal microbiota of conventional animals (including man) will often result in the elimination of the introduced bacteria.[2,23,30] At the end of the experiment the distribution of the donor, the recipient and the transconjugant *L. lactis* subsp. *lactis* populations was measured in four different sections of the intestine (duodenum, jejunum, caecum and colon).

MATERIALS AND METHODS

Animals

Conventional rats. Four male Wistar rats (approx. 10 wks old), obtained from Møllegaarden Breeding Centre, Denmark, were housed pairwise in stainless-steel wire cages. Feed (Altromin 1324 obtained from Brogaarden, Denmark) and acidified water were given *ad libitum*. The ambient room temperature was maintained at 22 ± 1°C with a light period from 9.00 p.m. to 9.00 a.m. The relative humidity was 55 ± 5%.

Germfree rats. Eight germfree Wistar rats (17–21 wks old), bred at the Institute of Toxicology, were originally obtained from Zentrallinstitut für Versuchstierzucht, Hannover, Germany. The germfree status of the rats was ensured before the start of the experiment by testing faecal samples for aerobic and anaerobic growth of bacteria and yeast. Group A consisted of four male rats and group B of two male and two female rats; Group A was used for cross 1 and group B for cross 2 (Table 2). The groups were kept in separate flexible isolators (Isotec type 12134, Olac, Oxford, UK). The rats were given 50 kGy irradiated feed (Altromin 1314) and 50 kGy irradiated drinking water *ad libitum*. All animals were housed individually in type III macrolon cages supplied with a grid and 50 kGy irradiated hardwood bedding (Red Special, Spanwell). Air-inlets as well as air-exhaust were supplied with Hepa filters (Interfilta limited, grade IFO). The ambient room temperature was maintained at 20 ± 1°C with a light period from 9.00 p.m. to 9.00 a.m. The relative humidity was approximately 55 ± 5%. Air was changed in the isolator between eight and ten times per hour.

Bacterial strains, plasmids and growth conditions

The strains and the plasmid used in this study are listed in Table 1. *L. lactis* subsp. *lactis* strains were stored at − 70°C in 15 per cent glycerol and propagated at 30°C in GM17 medium (M17

Table 1. Bacterial strains and plasmid

Strain, plasmid	Use in experiment	Characteristic(s)	Source or reference
Strains			
L. lactis subsp. *lactis*			
NCDO712		Wt	Gasson[11]
NCDO712 (pAMβ1)	Donor, cross 1	Wt containing conjugative plasmid pAMβ1; Em^r	
MG1363-1	Recipient, cross 2	Rif^r, Fus^r mutant of MG1363. A plasmid free derivative of NCDO712	F. Vogensen Royal Vet. and Agric. University of Denmark
MG1614	Recipient, cross 1	Plasmid free Str^r, Rif^r derivative of NCDO712	Gasson[12]
BLO1	Transconjugant	Transconjugant of MG1614 and NCDO712-pAMβ1; Rif^r, Str^r, Em^r	This study
BLO2	Donor, cross 2	MG1614 containing pAMβ1; Rif^r, Str^r, Em^r	This study
BLO3	Transconjugant	Transconjugant of MG1363-1 and BLO2; Rif^r, Fus^r, Em^r	This study
Plasmid			
pAMβ1		Conjugative plasmid from *Enterococcus faecalis*; Em^r	Clewell[4]

Wt, wild type; Em^r, erythromycin resistant; Fus^r, fusidic acid resistant; Rif^r, rifampicin resistant; Str^r, streptomycin resistant.

medium[28] where 0·5 per cent w/v glucose has replaced lactose). The antibiotics erythromycin, rifampicin, streptomycin, and fusidic acid were added to GM17 at final concentrations of 5, 50, 200 and 10 μg/ml, respectively.

The broad host range plasmid pAMβ1 used in this study has a size of approximately 17 MD (26 Kb).[4] It has been isolated from *E. faecalis* and confers resistance to erythromycin.[4]

Conjugation procedures

In vitro *conjugation.* Mating between *L. lactis* subsp. *lactis* strains was performed by a modification of the method of Mckay *et al.*[21] Recipient and donor were grown overnight in GM17 medium containing the relevant antibiotics and 5 μl of the donor and 5 μl of the recipient was mixed on GM17 plates. After incubation for 24 h at 30°C, the plate was washed with 10 ml saline (0·9 per cent NaCl) and the concentration of donors, recipients and transconjugants was determined by seeding 0·1 ml of serial dilutions on GM17 agar with relevant antibiotics and incubation at 30°C for 2 d. The donor–recipient pairs were NCDO712(pAMβ1) to MG1614 in cross 1 and BLO2 to MG1363-1 in cross 2 (Table 2).

In vivo *mating procedure.* Thirty millilitres of an overnight culture of the *L. lactis* subsp. *lactis* strain was centrifuged for 10 min at 2000 ***g*** at 4°C. The pellet was resuspended in 5 ml saline and the rats were dosed with 1 ml bacterial suspension (approximately 10^9 c.f.u./ml) intragastrically by gavage. The *L. lactis* strains used were the same as for the *in vitro* conjugation (Table 2). The bacterial concentration in the rat faeces was determined using the spread plate method. In cross 1, the recipient, donor and transconjugant strains were selected on GM17 supplemented with rifampicin+streptomycin, erythromycin, and rifampicin+streptomycin+erythromycin, respectively. In cross 2, the recipient, donor and transconjugant strains were selected on GM17 supplemented with fusidic acid, streptomycin+erythromycin, and fusidic acid+erythromycin, respectively. At the end of the experiment the rats were necropsied and the *Lactococcus* concentration was determined in the lumen

Table 2. Transfer of (pAMβ1) between *L. lactis* subsp. *lactis in vitro* per ml suspension, and *in vivo* per gram faeces

Cross	Donor	Recipient	Ratio of transfer[a]	
			In vitro[b]	End of experiment *in vivo*
	L. lactis subsp. *lactis*	*L. lactis* subsp. *lactis*		
1	NCDO712 (pAMβ1)	MG1614	$9{\cdot}2 \pm 0{\cdot}3 \times 10^{-1}$	$4 \pm 4 \times 10^{-5}$[c]
2	BLO2	MG1363-1	$2{\cdot}0 \pm 1{\cdot}0 \times 10^{-2}$	$2 \pm 3 \times 10^{-3}$[d]

Ratio of transfer expressed as mean ± SD.
[a]Ratio of transconjugants per recipient.
[b]Ratio of transfer is a mean of three experiments.
[c]Ratio of transconjugants per recipient at the end of experiment (mean of four rats).
[d]Ratio of transconjugants per recipient at the end of experiment (mean of three rats).

contents of five different sections of the intestine: duodenum, jejunum, caecum, colon and rectum.

Experimental design

Conventional rats. Four conventional rats were dosed with the recipient strain MG1363-1 and the donor strain BLO2 (MG1614(pAMβ1)) on four consecutive days. The faeces from the rats were examined daily from 1 d before dosing start until 18 d after the first dosing.

Germfree rats—cross 1. Initially the rats were dosed once with the recipient strain, MG1614. After a period of 29 d the donor strain NCDO712(pAMβ1) was dosed at experimental day 0 and again at days 6, 7, 8 and 9. The faecal samples were examined until day 37 by the spread plate method on GM17 medium with relevant antibiotics. Colonies, which were growing on the medium selective for donors, were tested at days 16, 21 and 28 to examine whether they were donors or transconjugants. The test was done by subculturing 50 colonies from each plate onto media selective for the transconjugant. Additionally plasmid screening was performed on some of these isolates. The rats were necropsied at day 37.

Germfree rats—cross 2. The recipient strain MG1363-1 and the donor strain BLO2 were dosed to the rats with few minutes interval at day 0. The faeces was examined until 10 d after dosing by the spread plate method on GM17 medium with relevant antibiotics. The rats were necropsied at day 18.

Plasmid stability in the donor was examined in cross 2 by direct plating on streptomycin plates (donor with or without plasmid) and compared with counts on streptomycin+erythromycin plates (donor with plasmid).

DNA investigations

Isolation and analysis of plasmid DNA. In order to verify the absence or presence of plasmid pAMβ1 inferred by the antibiotic susceptibility, 20 *L. lactis* subsp. *lactis* isolates, selected randomly from different rats, were screened for plasmids using the method of Anderson and McKay.[3] Agarose gel electrophoresis (0·7 per cent agarose) was performed in 0·5 × TBE buffer at 3 V/cm for 3 h according to Sambrook *et al.* (1989).[25] The gel was stained in 100 μg/ml ethidium-bromide for 20 min, and destained in distilled water for 20 min. In order to investigate the presence of the intact plasmid in the transconjugants plasmid DNA was isolated as described by O'Sullivan and Klaenhammer.[24] The plasmid DNA was analysed by restriction enzyme cutting with HinDIII (New England Biolabs, MA, USA) and the size and banding pattern were compared to plasmid DNA isolated from the donor.

Pulsed field gel electrophoresis (PFGE). PFGE was applied in order to ascertain the genotype suggested by the antibiotic susceptibility. The genomic DNA was prepared by a method adapted from the method described by Tanskanen *et al.*[27]

To prepare intact DNA in agarose plugs, cells were grown in 100 ml GM17 to an optical density of 0·6–1·0 OD_{578} units. Cells were harvested by centrifugation at 3000 *g*, washed in 100 ml 50 mM EDTA (pH 8·5) and pelleted. The cell pellet was kept at −20°C overnight and resuspended using 1·5 ml 50 mM EDTA (pH 8·5) for each OD unit per 100 ml culture harvested. Five hundred

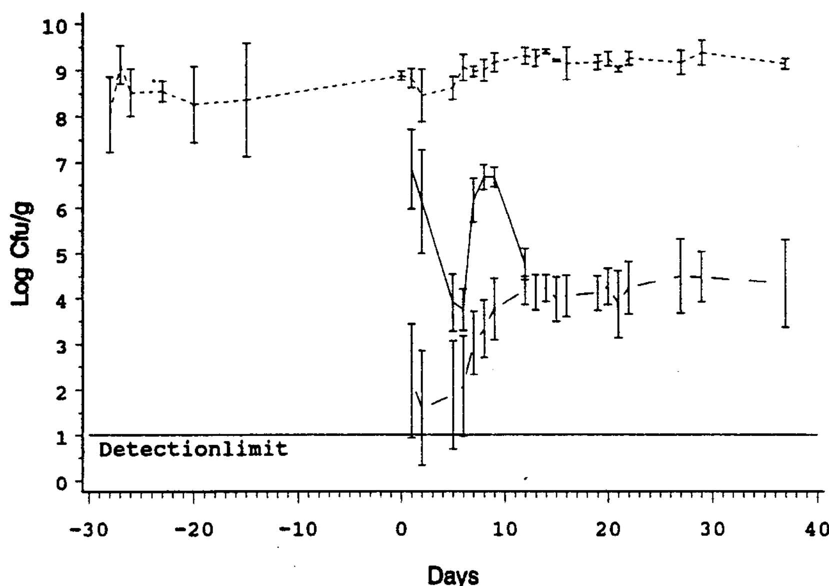

Figure 1. Mean concentration of *L. lactis* subsp. *lactis* strains in faecal samples from the four gnotobiotic rats of cross 1. The germfree rats were dosed with the recipient strain MG1614 (– – –) on day 29. The donor strain NCDO712(pAMβ1) (——) was dosed on day 0 and again on days 6, 7, 8 and 9. Concentration of the transconjugant BLO1 (— · —). The detection limit was generally 10 c.f.u./g, but for the donor strain the detection limit was 1·7 logarithms below the concentration of transconjugant (see Materials and Methods). Vertical bars indicate the standard deviation

microlitres of cell suspension were mixed with 3 ml SeaPlaque low melting agarose (FMC Bioproducts, Rockland, USA) at 47°C, pipetted into a Biorad plug mould and allowed to solidify at 4°C for 20 min. The plugs were incubated for 4–5 h in 3 ml lysozyme solution (2 mg lysozyme/ml 50 mM EDTA pH 8·5, 0·05 per cent N-lauryl-sarcosine). The DNA plugs were then transferred to a proteinase K solution (2 mg/ml in 10 mM Tris pH 8·0, 1 per cent SDS and 0·5 m EDTA) and incubated at 50°C overnight. The plugs were washed five times with 10 ml 50 mM EDTA (pH 8·5) at room temperature. A small slice of the plug (1–1·5 mm) was cut with a scalpel and incubated for 1 h at 4°C in 200 µl restriction enzyme buffer supplied by the manufacturer (Promega, Madison, WI, USA). The buffer was removed and replaced with 200 µl buffer containing 20 units SmaI restriction enzyme. The digestion was performed overnight at 30°C. Plugs were slipped into the slots of a gel of 1·1 per cent SeaKem GTG (FMC Bioproducts) agarose dissolved in 0·5 × TBE. PFGE was performed using a CHEF DR2 system (Bio-Rad, Sweden) for 21 h with switch times changing from 5 s to 40 s through the electrophoresis. The agarose gel was stained with ethidium-bromide (100 µg/ml), destained in 0·5 × TBE buffer and photographed.

RESULTS

In vitro *mating*

The *in vitro* transfer rate of plasmid pAMβ1 was found to be 9×10^{-1} (Table 2) when the donor strain was *L. lactis* subsp. *lactis* strain NCDO712(pAMβ1) and the recipient was strain MG1614 (cross 1). The transfer rate was slightly lower (2×10^{-2}) when the conjugation pair was MG1614(pAMβ1) as the donor and MG1363-1 as the recipient (cross 2).

In vivo *mating*

Conventional rats. Before administration of the test strains, faecal samples from the four conventional rats were examined and no strains of *L. lactis* were detected (detection limit: 10 c.f.u./g faeces). From 2 to 6 d after dosing, both the donor and recipient strains were found at levels of 10^3–10^4 c.f.u./g faeces. No strains of *L. lactis* were

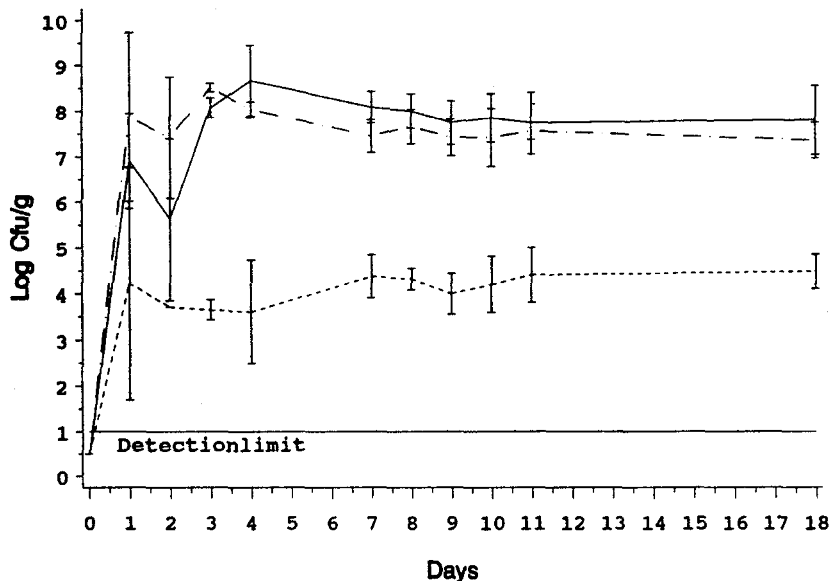

Figure 2. Mean concentration of the three populations of *L. lactis* subsp. *lactis* strains in faecal samples of three of the gnotobiotic rats of cross 2. In the fourth rat, the transconjugant concentration was approximately 2 logarithms lower until day 10 when it went below detection limit (data not shown). The rats were dosed with the recipient strain MG1363-1 (——) immediately followed by the donor strain BLO2 (— · —) on day 0. Concentration of the transconjugant strain BLO3 (- - -). The detection limit was 10 c.f.u./g. Vertical bars indicate the standard deviation

found later than 6 d after first dosing. With a detection limit of 10 c.f.u./g faeces, no transconjugants were found at any time in faecal samples from these animals (data not shown).

Germfree rats. When given to germfree rats, the recipient of cross 1, MG1614, established at a high level (10^8–10^9 c.f.u./g faeces). The donor strain, NCDO712(pAMβ1), dosed 29 d later (=day 0), was found in concentrations of 10^5–10^8 c.f.u./g faeces on day 1, but the concentration declined over the following 4 d (Figure 1). Therefore, the donor strain was re-administered to the animals on four consecutive days (day 6–9). This resulted in concentrations of approximately 10^6 c.f.u./g faeces of the donor for a few days, but then the concentration declined and the donor could not be detected after day 14 (Figure 1). Faecal samples were cultured every day during the initial period before the donor re-administration, and transconjugants were found in low concentrations (10–$10^{3 \cdot 8}$ c.f.u./g faeces) in two, three or all of the four animals at the respective sampling days. After re-administration a stable population of transconjugants established in all animals at a level of 10^4 c.f.u./g faeces. Colonies growing on the medium selective for transconjugants and donors after day 14 were found only to be transconjugants.

In cross 2, the recipient and donor strain, MG1361-1 and BLO2 respectively, were administered once to germfree rats. Both strains colonised the animals at a high level (10^7–10^8 c.f.u./g faeces) throughout the entire experimental period (Figure 2). Transconjugants were detected from 24 h after dosing of donor and recipient, and they remained at a constant concentration of 10^3–10^4 c.f.u./g faeces in three of the animals (Figure 2). The concentration of transconjugants in faeces of the 4th rat was 10^2 c.f.u./g faeces for 10 d and then went below the detection limit (10 c.f.u./g). Plasmid loss was not detected in the donor of cross 2 for the duration of the experiment. pAMβ1 becomes unstable in *L. lactis* subsp. *lactis* strains used in this study when grown *in vitro* at 37°C (results not shown). However, in the animals, no sign of instability of pAMβ1 was detected under the conditions prevailing in the rat intestine.

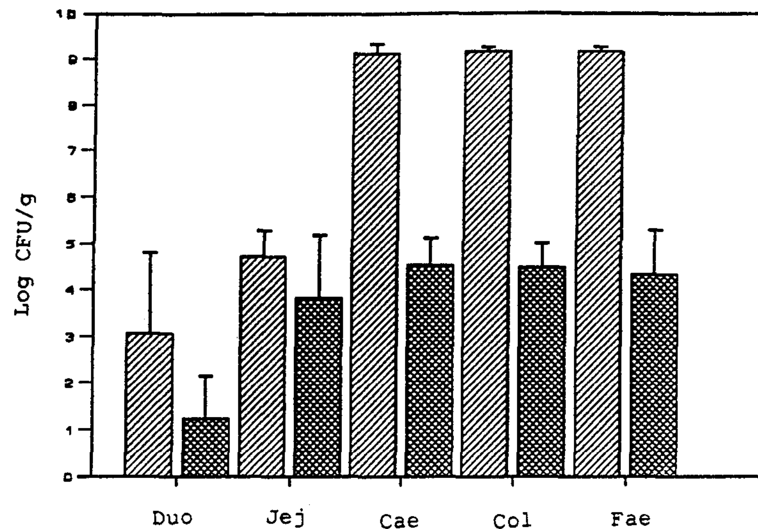

Figure 3. Distribution of the *L. lactis* subsp. *lactis* strains in the intestine of the four gnotobiotic rats of cross 1 at necropsy 37 d after dosing of donor strain. Mean concentration of recipient MG1614 (▨) and transconjugant strain BLO1 (▩) in the intestinal contents of duodenum (Duo), jejunum (Jej), caecum (Cae), colon (Col) and rectum (faeces; Fae). Detection limit was 10 c.f.u./g. Vertical bars indicate the standard deviation

Distribution of transconjugants in the intestine. The distribution of the *L. lactis* strains in the intestinal contents in cross 1 is illustrated in Figure 3. The recipient and transconjugant concentrations were of the same magnitude in jejunum, whereas the recipient concentration was 10^4–10^5 higher than the transconjugant in caecum, colon and faeces. By the methods applied donors were not found in any of the intestinal regions.

Likewise, the intestinal distribution for cross 2 is shown in Figure 4. Only results from the three rats where transconjugants were still present at the end of experiment are included in this figure; in correspondence with results from the faeces samples after day 10, the transconjugant concentration in the intestinal contents of the fourth rat was found to be below the detection limit (10 c.f.u./g). For the remaining three rats the concentrations of recipients, donors and transconjugants were all similar in the jejunum. Further down the digestive tract the concentrations of recipients and donors were 10^3–10^5 higher than the concentration of the transconjugants in caecum, colon, and faeces. It should be noted that in jejunum the recipient population was indistinguishable from the transconjugant population. This is a problem when the transconjugant concentration is similar to or greater than the recipient concentration, because both populations grow on the plates selective for recipients. However, it should also be noted that the recipient population is clearly present and distinguishable from the transconjugants in the duodenum (Figure 4).

Plasmid screening and pulsed field gel electrophoresis

Twenty *L. lactis* isolates from different rats were examined by plasmid screening to verify the absence or presence of plasmids inferred by the antibiotic susceptibility. The results showed that the transconjugants all contained a large plasmid of the same size as the one present in the donor (data not shown). The results of restriction enzyme digests of the parent plasmid pAMβ1 and the plasmids isolated from the transconjugants show an identical pattern, indicating that the plasmid transferred to the transconjugants has not rearranged (Figure 5). The size of transferred DNA is calculated to approx 26 Kb which corresponds to the reported size of pAMβ1[4] and is identical with the size of plasmid DNA isolated from the donor. The PFGE verified that 12 random isolates showed the strain-specific band pattern inferred by the antibiotic resistance (data not shown). In the cross 1 experiment the PFGE

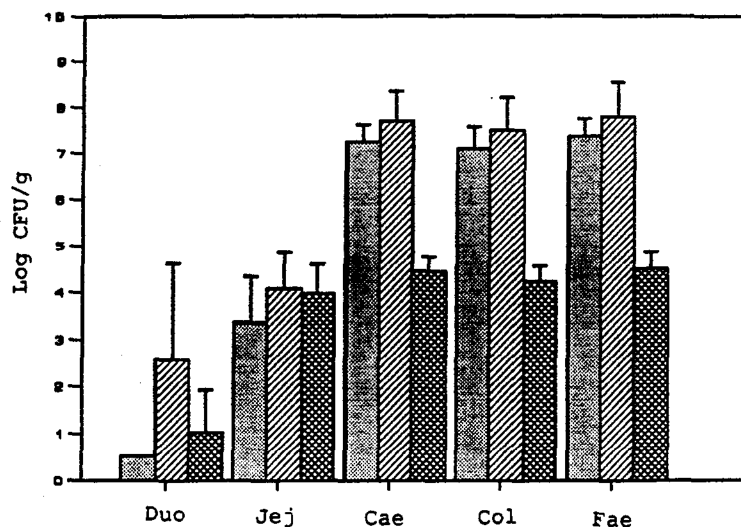

Figure 4. Distribution of the *L. lactis* subsp. *lactis* strains in the intestine of three of the gnotobiotic rats of cross 2 at necropsy 18 d after dosing of donor and recipient. In the fourth rat, the transconjugants were not found (data not shown). Mean concentration of the donor BLO2 (▨), recipient MG1363-1 (▨) and transconjugant BLO3 (■) in the intestinal content of duodenum (Duo), jejunum (Jej), caecum (Cae), colon (Col), and rectum (faeces; Fae). Detection limit was 10 c.f.u./g. Vertical bars indicate the standard deviation

verified the elimination of the donor and the establishment of the transconjugants.

DISCUSSION

The dosing of donor and recipient strains of *L. lactis* was done in a similar manner to conventional and germfree rats.

In conventional rats, the *L. lactis* strains introduced were quickly eliminated from the intestine of all animals. Clearly, colonisation defined as 'the detection of a microorganism for a relevant period of time at a constant level'[18] was not established by the test strains in conventional rats. This lack of colonisation was not unexpected, since it has been shown that the normal intestinal microbiota provides an excellent resistance against colonisation by introduced bacteria.[23,30,32] Even though donor and recipient do survive gastrointestinal passage, no transconjugants were retrieved in the faecal samples. However, the process of conjugation cannot be excluded. The lack of transconjugants could reflect a relatively low frequency of the process in combination with a low and declining concentration of the donor and recipient strains. By mathematical modelling and use of transfer rates obtained *in vitro*, Freter *et al.*[10] have shown that the expected concentration of transconjugants is usually well below the detection limit if the donor strain is eliminated within a few days.

In contrast, the use of germfree rats makes it possible to introduce and maintain specific bacterial populations in the intestine. The high degree of colonisation is due to the lack of a normal protective microbiota,[9] and thus the high concentrations of donor and recipient make it possible to detect transconjugants in faecal samples even if the frequency of conjugation is low.

In cross 1, the *L. lactis* subsp. *lactis* strain MG1614 colonised at about 10^9 c.f.u./g faeces. This is similar to the levels of previously reported colonisation of *L. lactis* in monoassociated mice.[14,15] The second test strain, *L. lactis* subsp. *lactis* strain NCDO712 (pAMβ1), introduced four weeks later was not able to establish a stable population in the intestine (Figure 1). This is also a typical situation, when an introduced bacterial strain has to compete with a well-established population of the same species.[7,17] However, because of repeated dosing, the donor strain was detectable in faeces for a period of 12 days, after which it dropped below detection limits. In contrast to this the level of transconjugants (MG1614 with the plasmid pAMβ1) was constant for the

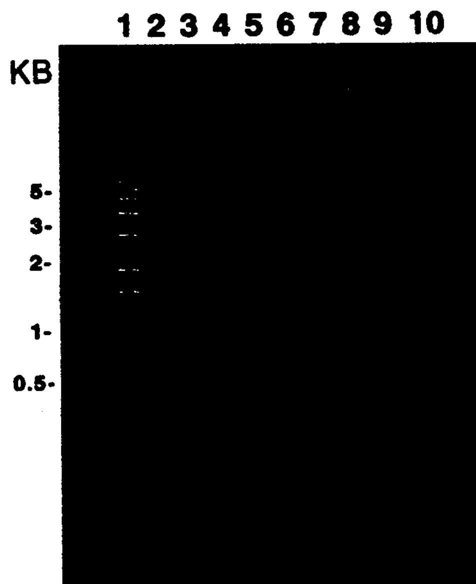

Figure 5. Agarose gel electrophoresis of plasmid DNA isolated from donor and transconjugants digested with HindIII to confirm the transfer of the intact plasmid without modification to the transconjugants. Lane 1, Gibco BRL 1 Kb ladder; lane 2, donor, MG1614(pAMβ1); lanes 3–10, transconjugants isolated from the rat intestine

experimental period, i.e. up to 18 days after the last dosing with the donor. Interestingly, conjugation took place even though the donor strain did not colonise, and the produced transconjugants were able to colonise the intestine. A likely explanation for the ability of the transconjugants to colonise could be that a passing donor conjugates with an established recipient and establishes a transconjugant population either by transconjugant growth or by transconjugant to recipient transfer or by a combination of these.

When given simultaneously in two separated single doses, the two strains of cross 2 (*L. lactis* subsp. *lactis* MG1614[pAMβ1] and MG1363-1) established and maintained constant populations at levels of 10^8 c.f.u./g faeces (Figure 2). There was no exclusion of either of the two strains, which reflects their equal fitness for the growth conditions in the germfree intestine. Transconjugants were detected at a concentration level approximately 10^4 c.f.u./g below the donor and recipient level, and these levels were constant in three of the four rats up to 18 days after dosing at which time the animals were killed. As in cross 1, the transconjugants of cross 2 were able to maintain a stable population of 10^3–10^4 c.f.u./g faeces and thereby form a third population. However in cross 2 both the donor and recipient strains were present simultaneously in the intestine, and therefore the population of transconjugants in this experiment could result from a constant combination of donor to recipient conjugation, transconjugant to recipient conjugation and growth of the transconjugant.

Some natural and genetically engineered plasmids become unstable in the digestive tract.[15] In this study the pAMβ1 plasmid was also shown to be unstable in the donor BLO2 when grown *in vitro* at 37°C for 55 generations in GM17 medium (results not shown). In the animals, however, there was no sign of plasmid instability when examined by direct plating of the faecal samples or at least such instability did not result in the competitive exclusion of the plasmid-containing populations.

In both experiments, the concentration of the various *L. lactis* subsp. *lactis* strains in the large intestine of animals reflected the concentrations detected in the faecal samples in the last part of the experimental period, i.e. the concentration of transconjugants was approximately 10^4 c.f.u./g lower than the concentration of the recipient strain (Figures 3 and 4). The concentration of transconjugants in both cross 1 and 2 reached 10^4 c.f.u./g in the small intestine and remained at this level throughout the large intestine. In contrast the concentration of recipients in cross 1 and recipient and donor in cross 2 increased from 10^4 c.f.u./g in the small intestine to 10^9 c.f.u./g in the large intestine. The most likely explanation to this would be that the transconjugants did have a niche in the small intestine, whereas they were only transiently present in the large intestine. Assuming that the conjugation frequency is of the same order of magnitude in the small and the large intestine the pAMβ1 plasmid seems to have conveyed to the recipient a competitive advantage in the small but not in the large intestine.

The *in vitro* transfer ratios of crosses 1 and 2 were comparable to the transfer ratios measured in the jejunum, but 10^3–10^5 times higher than the *in vivo* ratios measured in the faecal samples. The *in vitro* transfer ratios in crosses 1 and 2 are nearly identical with the 5×10^{-1} transconjugants/recipient reported by Gasson and Davies[13] with NCDO712 derivatives in filter mating experiments.

The significant difference between the transfer ratio measured in faeces and in the jejunum indicates that conclusions based on data from faecal samples alone may not represent the

situation in the intestine of the animal and conclusions regarding transfer frequencies and growth rates must therefore be supported by additional experimental data, e.g. spatial distribution of the donor, transconjugant and recipient populations.

In more general terms the results from these experiments seem to underline the potential of a plasmid to colonise the intestine, even though the accompanying donor microorganism is eliminated over time.

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Monitoring of Genetically Modified *Lactococcus lactis* in Gnotobiotic and Conventional Rats by Using Antibiotic Resistance Markers and Specific Probe or Primer Based Methods

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Summary

Germ-free as well as conventional rats were dosed by proteinase-deficient *Lactococcus lactis* Bu-2–60 as a potential recipient strain to monitor colonization and lateral gene transfer. *Lactococcus lactis* MG1820 (pLMP1), *Lactococcus lactis* MG1820 (pAMβ1), or *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) were applied as potential donor strains in different experiments. *Lactococcus lactis* MG1820 (pLMP1) contained a genetically modified proteinase gene, whereas *Lactococcus lactis* MG1820 (pAMβ1) carried the highly conjugative broad host-range plasmid pAMβ1. *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) was constructed as a potential donor for both plasmids. Faecal and intestinal samples were analysed for the presence of the initially introduced strains and for lateral gene transfer of the modified proteinase gene and/or the plasmid pAMβ1 by applying conventional antibiotic resistance based screening techniques, *in situ* colony hybridization with specific probes, and diagnostic polymerase chain reaction. The germ-free rats were readily colonized by all administered strains, whereas the conventional rats were colonized only transiently. Lateral transfer could not be observed for the modified proteinase gene. However, the plasmid pAMβ1 was transferred to the lactococcal recipient strain and in one case to a strain or close relative of *Enterococcus faecalis* of the indigenous intestinal flora of a conventional rat.

Key words: Diagnostic PCR - Genetically modified organisms -Gnotobiotic rat - Intestinal tract - *Lactococcus lactis* - Monitoring studies - Proteinase gene - Specific probes

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Introduction

Lactic acid bacteria play a central role in a variety of food fermentation processes. They are of particular importance in the preparation, processing and preservation of dairy products. The composition, fitness, and physiological capabilities of the microbial flora present during milk processing substantially influence the flavour, taste, and perishability of the particular products. Consequently, the inoculation of milk and milk products with properly selected and carefully maintained microorganismal cultures or communities as starters is standard in the dairy industry. During the history of starter culture development major efforts had been directed to improve the stabilities of these communities with respect to their composition and product relevant physiological properties, to tailor and/or enhance quality and performance of the capabilities of the starter organisms in favour of a better product, and to adopt the starter bacteria to environmental conditions and stress as a consequence of established or newly modified processing techniques.

Given the progress in the study of genetics of lactic acid bacteria and the availability of modern methods of gene technology, systematic and directed modification of starter bacteria nowadays is possible. Moreover, these modifications cannot only be introduced in a target-directed way, but also their location and nature on the genomic material can directly be controlled and monitored by sequencing and specific probe hybridization and/or specific *in vitro* amplification approaches. Especially the monitoring aspects are of critical importance in the context of a responsible construction and release of genetically modified starter bacteria within the scope of national and international regulations and the conditions of the particular cases. Recent studies (*Hertel* et al., 1992) have shown, that the use of hybridization probes targeted to genetically modified nucleic acid regions allows reliable monitoring of the modification itself as well as of the organisms carrying the respective modification. Silent mutations were introduced into a naturally occurring proteinase gene (*De Vos* et al., 1989) without altering the predicted amino acid sequence and the activity of the corresponding gene product (*Hertel* et al., 1992). Moreover, it was demonstrated that the use of specific probes for the original and altered genes in combination with rRNA targeted species or group specific probes (*Schleifer* et al., 1993; *Amann* and *Ludwig*, 1994; *Amann* et al., 1995) allows to identify the gene variant as well as its host organism by applying *in situ* colony hybridization (*Hertel* et al., 1992).

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Culture conditions. Lactococci were grown aerobically at 30°C in M17 medium (*Terzaghi* and *Sandine*, 1975) supplemented with 0.5% glucose (w/v). *Lactobacillus curvatus* was cultivated under same conditions in MRS broth (*De Man* et al., 1960). CATC agar (*Reuter*, 1968) was used for the selective aerobic cultivation of enterococci at 37°C. Screening for lactose positive strains was done by growing them on bromcresolpurpur lactose indicator agar (*McKay* et al., 1972). The strains were analysed for antibiotic resistances by using the

same medium supplemented with appropriate combinations of chloramphenicol, erythromycin, rifampicin and streptomycin (5, 50, 100, and 500 mg/l, respectively).

Conjugation. Conjugation experiments with lactococcal strains were performed by using the method of *McKay* et al. (1980) with slight modifications. Recipient and donor strains were grown overnight in M17 broth supplemented with the respective antibiotics. Cell suspensions of the donor and recipient strains were mixed (1 : 2, v/v), and total volumes of 200 μl of the resulting suspensions were plated on GM17 agar (M17 supplemented with 0.5% glucose). The plates were incubated anaerobically at 30°C for 18h. The cells were resuspended by rinsing the plates with 1 ml of GM17 broth containing the corresponding antibiotics.

Transformation. Transformation of lactococcal strains with plasmid DNAs was achieved by electroporation as described earlier (*Hertel* et al., 1992).

Animals. A total of 30 germ-free Wistar rats bred at the Institute of Toxicology (originally obtained from Zentralinstitut für Versuchstierzucht Hannover, Germany) were kept in three groups. Each group consisted of 5 males and 5 females. The rats in group A and B were all 13 weeks old, whereas the rats in group C were 20 weeks old at the beginning of the experiments. Additional groups D and E each consisted each of 5 Mol:Wist conventional male rats 16 weeks old from Møllegaard Breeding Center Ltd. (Danmark). The animals were kept and feeded as described previously (*Schlundt* et al., 1994).

Dosing of the animals. Lactococcal cells were harvested from liquid overnight cultures by centrifugation (2000 $\times$ g; 10 min) at 4°C. The cells were resuspended in the initial volume of saline supplemented with 0.1% peptone. The individual rats were dosed intragastrically by a serving of 1 ml cell suspension of the potential donor strain. The potential recipient strains were applied to the initially germ-free rats one or two weeks later. In the case of conventional rats, the dosage of the second strain was 15 min after the first. The numbers of colony forming units of lactococci given to the animals are listed in Table 2.

Bacterial sampling. Faecal samples (1 g) were taken daily and homogenized in saline supplemented with 0.1% peptone (9 ml). Tenfold serial dilutions were prepared in the same buffer and samples were applied to the appropriate selective media. Different groups of the rats (Table 2) were necropsied 1, 2, or 3 weeks after the application of the recipent strains. Samples from the lumen contents of six different sections of the intestine (duodenum, jejunum, ileum, caecum, colon and rectum) were treated similarily for selective quantification.

Identification of strains. The identities of bacterial strains were evaluated applying the API test systems.

Statistical analysis. Bacterial counts were statistically analysed using Paradox 3.0 (Borland International Inc., Scotts Valley, CA, USA) and Quattro Pro 4.0 (Borland International Inc., Scotts Valley, CA, USA). Graphs were made using the Jandel Scientific Graphic System ("SigmaPlot", Erkrath, Germany).

Purification of DNA. Genomic DNA from pure cultures was isolated according to *Marmur* (1961). Plasmid DNAs were purified as described by *O'Sullivan* and *Klaenhammer* (1993). DNA extraction from faeces and intestine contents was performed according to *Hertel* et al. (1991) with some modifications. The samples were suspended in 9 volumes of saline supplemented with 0.1 % (w/v) peptone. Glass beads (ø 0.18 mm), 50 μl 25% SDS and 600

μl Phenol/Chisom (Chloroform:Isoamylalcohol, 24:1) were added to 1ml of the suspension. The mixture was sonicated for 4.5 min and subsequently incubated at 90°C for 10 min. A further extraction of the aqueous phase by phenol (saturated in H_2O) was followed by ethanol precipitation of the nucleic acids.

In vitro DNA amplification. In vitro amplification of proteinase *prtP* and/or *prtP'* gene fragments was achieved by using the primers specified in Table 3 and performing 35 cycles of incubation at 94°C, the annealing temperature specified in Table 3, and 72°C for 1 min, 1.5 min, and 2 min, respectively.

Labelling of probes. Radioactive 3'-end labelling of the oligonucleotide probes was performed by using terminal deoxynucleotidyltransferase (Boehringer, Mannheim, Germany) in combination with [α-^{32}P] CTP. Digoxigenin-ddUTP and the 3'-end Labelling Kit of Boehringer (Mannheim, Germany) were used for non-radioactive labelling.

Hybridizations. In vitro amplified DNA fragments were transferred to Zeta probe me-branes (Biorad, München, Germany) either by Southern transfer (*Southern*, 1975) using a vacuum transfer unit from Serva (Heidelberg, Germany) or by direct application using a dot blot apparatus (Minifold, Schleicher & Schuell, Dassel, Germany).

The preparation of the membranes for colony hybridization was performed as described previously (*Tatzel* et al., 1994) with some modifications. The incubation time for growing the lactococci on the nylon membranes was 40 hours. The lactococci were grown either directly on the membrane or alternatively transferred from M17 agar to the membranes by colony lifting followed by further incubation on M17 agar complemented with 100 U/ml penicillin G. The lysis solution was prepared without mutanolysin and the filters were incubated on 0.2N NaOH, 5% SDS soaked filter paper (Whatman 3MM) for 15 min at room temperature before heating them in the microwave oven.

Hybridizations to the probes listed in Table 3 were carried out as described previously (*Hertel* et al., 1992; *Tatzel* et al., 1994) at hybridization and washing temperatures specified in Table 3.

Results

Construction of recombinant lactcocci

Lactococcus lactis MG1363 was transformed by electroporation with DNA of the recom-binant plasmid pLMP1 (*Hertel* et al., 1992) carrying the modified proteinnase gene *prtP'*. The resulting strain *Lactococcus lactis* MG1363 (pLMP1) received plasmid DNA of pAMß1 purified from *Lactobacillus curvatus* LTH 1704 (*Vogel* et al., 1992) by electroporation. The conjugative plasmid pAMβ1 was transferred by surface mating from *Lactobacillus curva-tus* LTH 1704 (*Vogel* et al., 1992) to *Lactococcus lactis* MG1820 (pLMP1) containing the modified proteinase gene *prtP'* located on a plasmid (pLMP1; *Hertel* et al., 1992). The re-sulting strain *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) contained both plasmids. The transfer frequencies were low (5×10^{-8} per recipient). *Lactococcus lactis* MG1363 (pLMP1, pAMß1) carrying both plasmids served as donor for the proteinase-negative *Lactococcus lac-tis* MG1820 (*Maeda* and *Gasson*, 1986) to receive the plasmid pAMβ1 (*LeBlanc* and *Lee*,

1984) by conjugation as *Lactococcus lactis* MG1820 (pAMβ1). Transfer frequencies of 1.3 × 10^{-1} were found after surface mating. It was proven by Southern and *in situ* colony hybridizations with pLMP1- and pAMß1-specific probes (PLC2 and PAM, respectively) that the transconjugant did only receive the plasmid pAMß1.

Design of specific probes and primers

The specific probes PLC1 and PLC2 for the original and the modified proteinase genes, respectively, have been described elswhere (*Hertel* et al., 1992). A pair of primers PLC3 and PLC4 specific for target sites within the proteinase *prtP* gene were designed (Table 3) and their specificities evaluated by Southern and dot blot hybridizations to purified plasmid and genomic DNAs from lactococci and lactobacilli. In addition, a specific probe (PAM; Table 3) for the plasmid pAMβ1 was constructed and its specificity evaluated by Southern and colony hybridizations to purified plasmid DNA (pAMß1) or cells of *Lactococcus lactis* MG1363 (pLMP1, pAMβ1), respectively.

Design of a diagnostic PCR approach

The 908 bp gene fragment which is amplified using the primer pair PLC3 and PLC4 contains the site of silent mutations (*Hertel* et al., 1992; Figure 1). The fragments which are amplified from the original as well as from the modified gene served as target molecules for diagnostic hybridizations to the gene variant specific primers PLC1 (wild-type) and PLC2 (modified gene).

Alternatively, the gene-specific amplification primer PLC3 was used in combination with either the wild type (PLC1) or modification specific primer (PLC2; Figure 1). Amplification products of 605 base pairs were only detectable after agarose gel electrophoresis when the respective target sites were present in the sample (Figure 2). A second diagnostic PCR-based technique allowed the simultaneous amplification of the large (908 bp) fragment resulting from proteinase gene specific amplification (PLC3 and PLC4) and the smaller fragment resulting from modification or wild-type specific amplification with PLC3 and either PLC1 or PLC2 (Figures 1 and 3). In order to obtain comparable amounts of the amplification products, the optimum primer concentrations had to be experimentally determined. 50 pmol of the modification and wild type specific primers (PLC1 and PLC2) as well as of the proteinase gene specific primer PLC3 had to be used whereas only 1 pmol of the second proteinase gene specific primer PLC4 had to be added.

The three methods were used for the analysis of complex samples such as milk, intestine contents, and faeces. Lactococci were grown in reconstituted skim milk overnight. The original samples were tenfold seriously diluted and the cells in 1 μl fractions were directly subjected to the PCR assays. Amplified DNA fragments could be visualized after gel electrophoresis by ethidium bromide staining. The presence of 10^2 - 10^3 cells per diluted sample was necessary to obtain detectable ammounts of amplified DNA. The detection limit of the PCR technique was also studied by adding known amounts of lactococci to faecal samples. 500 mg samples were used as source of nucleic acids. 10^4 c.f.u. in 500 mg faeces could be detected applying the combination of PCR amplification and subsequent specific probe hybridization.

In vivo mating studies

In vivo mating studies were performed using conventional and germ-free rats (Table 2) to see whether the plasmid-encoded genetically modified proteinase gene could be laterally transferred under the environmental conditions of the rat intestine. The proteinase-negative *Lactococcus lactis* Bu-2–60 (*Neve* et al., 1984) was used as a potential recipient in all experiments. Three strains of *Lactococcus lactis* MG1820 which differed with respect to their plasmid contents were used as potential donor strains (Table 1). *Lactococcus lactis* MG1820 (pLMP1) carried the plasmid with the modified proteinase gene (*Hertel* et al., 1992). *Lactococcus lactis* MG1820 (pAMβ1) containing the highly conjugative broad host-range plasmid pAMβ1 was included as a reference. *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) which contains both plasmids was constructed to study whether the plasmids interfere with respect to lateral transfer. pAMß1–associated mobilization of proteinase plamids from lactococci has been reported by *Hayes* et al. (1990). Three groups (A,B, and C; Table 2) of germ-free rats, each comprising 10 individuals, were dosed with the recipient strain *Lactococcus lactis* Bu-2–60. As a second dosage *Lactococcus lactis* MG1820 (pLMP1), *Lactococcus lactis* MG1820 (pLMP1, pAMβ1), or *Lactococcus lactis* MG1820 (pAMβ1), were given. Two groups of conventional rats (D and E; Table 2), each comprising 5 individuals, were treated similarly with *Lactococcus lactis* Bu-2–60 first, and with *Lactococcus lactis* MG1820 (pLMP1) or *Lactococcus lactis* MG1820 (pAMβ1) later. The dosages, times of application, and specifications of the animals are given in Table 2.

After the application of the potential donor strains, faecal samples were taken daily from each animal and screened by spread plating on solid media supplemented with the appropriate antibiotics (Table 1; Figures 4 - 6). The initially germ-free rats of groups A and B and the conventional rats of groups D and E were necropsied two weeks after the dosage of the potential donor strains. The necropsy of the animals of group B was performed ten days after the second dosing. The samples from different parts of the intestine were also analysed by screening for antibiotic resistances (Table 4). Rifampicin and streptomycin were used to screen for the resistant recipient strain. Erythromycin and chloramphenicol were selective for the donor strains *Lactococcus lactis* MG1820 (pAMβ1) and *Lactococcus lactis* MG1820 (pLMP1), respectively. The potential donor strain *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) is resistant to the latter drugs. The appropriate combinations of the different antibiotics were used to search for potential transconjugants (Figures 4–6; Table 4). The data obtained by conventional analyses were evaluated by performing diagnostic PCR and/or *in situ* colony hybridizations with selected samples.

Group A. The germ-free rats were dosed with 3.1 $\times$ 10^9 colony forming units (c.f.u.) of the recipient *Lactococcus lactis* Bu-2–60. A rather constant concentration (about 10^9 c.f.u./g) of streptomycin- and rifampicin-resistant bacteria within faecal samples (Figure 4) indicated the effective colonization of the rats by this strain. One week after the first dosage, 3.2 $\times$ 10^9 c.f.u. of the potential donor *Lactococcus lactis* MG1820 (pLMP1) were given to the animals. The corresponding numbers of colonies/g faeces on chloramphenicol supplemented media were arround 10^7 - 10^8 indicating that the donor strain was also capable to colonize. No resistant cells were found on media containing all three antibiotics. No transfer of resistance genes occurred at detectable levels within the two weeks period of the experiment.

Faecal samples from two rats taken at the date of necropsy were analysed by *in situ* colony hybridization to the probe PLC2 which is specific for the modified proteinase gene. No hybridization was found with colonies grown on the streptomycin and rifampicin (chromosomal resistances of the recipient) supplemented media.

A ratio of about 10:1 of colony forming units of putative recipient and donor strains was found analysing the samples taken from the different parts of the intestine after necropsy of the rats. This correlation was in agreement with that obtained from the faecal samples. However, there was an 10^5 fold increase of the total numbers of c.f.u. observed along the digestive tract from the duodenum to the colon (Table 4).

Group B. The potential donor strain, which was applied to the rats of group B, *Lactococcus lactis* MG1820 (pLMP1, pAMβ1) contained the plasmid pLMP1 carrying the modified proteinase gene and in addition the conjugative broad host-range plasmid pAMβ1. Despite the later (two weeks after first dosage) application of the donor, total and relative colony numbers on chloramphenicol and erythromycin (plasmid conferred resistances of the donor) as well as on rifampicin and streptomycin (chromosomal resistances of the recipient) supplemented media were in the same ranges as found for the corresponding faecal samples from group A (Figures 4 and 5). In addition, approximately 10^4 c.f.u./g faeces could be grown on media containing the three antibiotics rifampicin, streptomycin and erythromycin indicating lateral gene transfer of pAMβ1 or at least of the erythromycin resistance gene to *Lactococcus lactis* Bu-2–60. No colonies of a phenotype exhibiting the simultaneous resistance to rifampicin, streptomycin, erythromycin and chloramphenicol could be found. No lateral transfer of the chloramphenicol resistance gene located on pLMP1 (modified proteinase) occurred at detectable levels.

At the date of necropsy, the duodenum, jejunum, and ileum contents of five rats were analysed by *in situ* colony hybridization to the pAMβ1 specific probe (PAM). The fraction of transconjugants detected among the colonies of the receptor strain grown on the rifampicin and streptomycin (chromosomal resistances of the recipient) supplemented plates was in the same range as found using conventional plating techniques and screening for antibiotic resistances (approximately 1:3, 1:30, and 1:100).

The relative and total numbers of colony forming units of the donor and recipient strains were in agreement with the results obtained from the different intestine samples from group A as derived from resistance data. Interestingly, the concentration of around 10^4 c.f.u. / g of transconjugants was rather constant in all regions of the intestine except the duodenum (Table 4). No indications of transfer of pLMP1 could be observed.

Group C. In the experiments with rats of group C, *Lactococcus lactis* MG1820 (pAMβ1) was appplied to the conventional rats as potential donor strain. The results obtained from the analysis of the animals of group C (Figure 6) supported the corresponding data for rats of group B. The numbers of transconjugants which were observed using the rifampicin, streptomycin and erythromycin resistance markers were in the same range in both experiments (approximately 10^4 c.f.u. / g faeces). A similar situation was found regarding the donor strains. However, the total numbers of recipients were slightly lower in the faeces of the rats of group C (appr. 5×10^8 c.f./g).

The bacterial concentrations in the different regions of the intestine also correlated well with those found for rats of groups A and B (Table 4).

Group D. The five conventional rats of group D were dosed with 8.3 × 10^8 c.f.u. of the recipient strain. After 15 min, 1.4 × 10^9 c.f.u. of the pAMβ1 containing *Lactococcus lactis* MG1820 (pAMβ1) were applied to the animals gastrointestinally. Faecal samples were taken daily, from one rat twice a day. The resistance patterns of the donor and recipient strains could be observed no longer then the second day after dosage.

After selective enrichment for enterococci, from one animal colonies of erythromycin resistant bacteria could be detected on the first day after dosage. Before the application of the donor strain, no erythromycine- or chloramphenicol-resistant bacteria could be grown on selective media for enterococci from faecal samples. The erythromycine-resistant organism could be identified as a close relative of *Enterococcus faecalis* by *in situ* colony hybridization to a specific rRNA targeted probe (DB8; *Betzl* et al., 1991). A second hybridization to the pAMβ1 specific probe (PAM) proved that lateral gene transfer had occurred from the introduced *Lactococcus lactis* MG1820 (pAMβ1) to a member of the indigenous enterococcal flora of the animals intestine.

Group E. The experiment with rats of group E was carried out in the same manner as that with the group D rats. The donor was replaced by *Lactococcus lactis* MG1820 (pLMP1) containing the plasmid-encoded modified proteinase gene. Again, recipient and donor could be detected in faecal samples during the first two days after dosage by selective cultivation on media supplemented with streptomycin and rifampicin or chloramphenicol, respectively.

The double samplings of the first four days from one animal were also analysed by *in situ* hybridizations of colonies grown on chloramphenicol supplemented media and by diagnostic PCR for the presence of the modified proteinase gene *prtP*. 1.5×10^4 and 2×10^2 c.f.u. of the potential donor strain were obtained on chloramphenicol supplemented plates 24 and 30 hours after its application, respectively. No colonies of chloramphenicol-resistant lactococci could be grown from feacal samples taken 48 hours after the dosage of the donor or later. The presence of target DNA could be demonstrated for the first two samples (24 and 30 hours after second dosage) by the gel electrophoretic analysis of specifically *in vitro* amplified proteinase gene fragments. Southern hybridizations of the amplified DNAs to the modification specific probe PLC2 revealed the presence of targets also in the third sampling (48 hours after second dosage).

Discussion

In vivo colonization and mating studies

Colonization. It was shown recently (*Gruzza* et al., 1992; *Schlundt* et al. 1994) that lactococci were able to stably colonize the intestine of germ-free rats. The strains as well as their plasmids persisted in the gut and could be detected in faecal samples at high titers. The subsequent administration of two different strains at short intervals always resulted in a higher fraction (10 : 1) of the first strain within the lumen contents of the different sections of the intestine and the faeces. One of the objects of the present study was to see whether similar results could be obtained applying alternative strains focussing on *Lactococcus lactis*

MG1820 (pLMP1). This strain was constructed as part of a model system for the development of detection methods for the monitoring of genetically modified microorganisms especially in the context of their application in foods and food production (*Hertel* et al., 1992). Given that genetically modified organisms can be intrinsic components of food and feed, studies on colonization of the intestine and persistance of the organisms while and after passing the consumer are of central interest.

Comparable high numbers of c.f.u. as described by *Schlundt* et al. (1994) were found for the sections of the intestine and the faecal samples by spread plating the samples on strain diagnostic media (antibiotics). The persistance of the recombinant plasmid (pLMP1) carrying the modification was also demonstrated by specific hybridization and diagnostic PCR techniques. This corroborates earlier findings (*Gruzza* et al., 1990; *Gruzza* et al., 1992; *Schlundt* et al., 1994) of colonization by genetically modified bacteria over time periods of at least four weeks.

Germ-free animals were used to study the maximal colonization potential of genetically modified microorganisms. The results obtained from such studies may reflect a "worst case" situation with respect to the potential impact of genetically modified organisms used in food processing and released with the products. Therefore, the strains were also applied to conventional rats where they had to interact and compete with the autochthonous flora. The intestine of these animals represents a more realistic model system for the study of the fitness of ingested genetically modified microroganisms. Only transient colonization for two or three days could be demonstrated by using conventional as well as probe- or PCR-based techniques. This indicates a low competitional potential of strains normally not present in the intestine. However, this assumption cannot be generalized and has to be checked for the particular organisms and especially the genetically engineered constructs. Similar results were obtained by Klijn et al. (1995). In a human feeding study, they were able to recover a genetically labelled *Lactococcus lactis* strain and to detect genetically labelled DNA from faecal samples within 3 and 4 days after oral application, respectively.

Lateral gene transfer. A major topic that is frequently discussed in the context of the release of genetically modifed organisms in general and, in particular, their application in food or food production is potential lateral transfer of the genetic modification. Even if the donor organism would not be capable to stably colonize the intestine, the modified genetic material could be laterally transferred to members of the indigenous flora. Lateral transfer of the conjugative broad host-range plasmid pAMβ1 between lactococcal donor and recipient strains within the gut of initially germ-free rats occurs at high rates, and the recombinant strains colonize the intestine at lower numbers together with the initally applied strains. The results obtained in the present study concerning the transfer of pAMβ1 supported those presented by *Guzza* et al. (1992) as well as by *Schlundt* et al. (1994) and were used as standard for the corresponding experiments using the model strain containing the plasmid encoded (pLMP1) modified proteinase gene. Furthermore, pAMß1 was included to study whether its described capability to mobilize the conjugal transfer of lactococcal plasmids (*Hayes* et al., 1990) would potentially facilitate the lateral transfer of the plasmid encoded modified proteinase gene *prtP'*. No transfer of plasmid pLMP1 could be observed in any

of the experiments by applying conventional screening for antibiotic resistances or specific probing techniques regardless whether the potential donor strain contained pAMß1 or not.

Interestingly, in one case of conventional rats, lateral transfer of pAMβ1 to an *Enterococcus* from the indigenous bacterial gut flora was detected by plating on selective agar supplemented with the appropriate antibiotics. The identity of the plasmid as well as that of the organisms were elucidated by specific hybridization probes. The hybridization to the rRNA targeted specific probe DB8 (*Betzl* et al., 1991) characterized the organism as a strain or close relative of *Enterococcus faecalis*. Similar results obtained by using conventional screening methods have been described earlier (*Gruzza* et al., 1992). These findings indicate that lateral transfer of genetic material among permanent and transient members of the gut flora via conjugative plasmids may readily occur. On the other hand, the failur to detect transfer of pLMP1 indicates different probabilities and frequencies for different recombinant constructions.

Applicability and detection limits of the nucleic acid based monitoring methods

Independently from the question whether lateral gene transfer within the gut may represent a risk or not, in general or in the particular case of a given genetic construction, detection systems for monitoring have to be applicable for the analysis of complex samples such as food, gut contents, and faeces. The model system consisting of lactococcal strains and proteinase *prtP* gene variants which had already been used for the analysis of mixed cultures and inoculated milk (products) (*Hertel* et al., 1992) was tested for its applicability in the scenario of the present study. The advantages of the *in situ* colony hybridization techniques over the conventional indirect detection methods based on the testing for antibiotic resistances as characteristics of a genetic modification and/or its host are their direct targeting of the genetic material itself. Antibiotic resistance markers may be lost independently of the genetic modification or may be transferred from the autochthonous flora. This problem does not exist if specific probes targeted to the modification itself are used for monitoring. The combined use of taxon- and modification-specific probes and *in situ* hybridization techniques, currently is the only method which allows to directly monitor lateral gene transfer in complex samples by combining organismal identification and detection of the modification (*Hertel* et al., 1992). This was convincingly demonstrated by the detection of lateral transfer of pAMβ1 from *Lactococcus lactis* MG1820 (pAMβ1) to *Enterococcus faecalis* as described above. As shown for the experiments with germ-free rats, the presence of the targets located on the respective plasmids in donors and transconjugants could readily be monitored by *in situ* colony hybridizations.

However, there are a few limitations of the method concerning its applicability and sensitivity. Only organisms for which suitable cultivation procedures are available can be studied. With genetically engineered micororganisms this is usually not a problem. However, if lateral gene transfer should be monitored in environmental samples there may be a large fraction of so far non-culturable organisms. Further problems which were evident studying the intestinal and faecal samples from conventional rats without antibiotic based preselection result from different growth requirements of the members of complex communities. It is often difficult to grow the different organisms to small colonies of comparable sizes and to

apply lysis techniques which are effective for all colonies. Parallel incubations of multiple membranes at different conditions help to reduce these problems.

In principle, *in situ* colony hybridization is a highly sensitive detection method. Using the appropriate dilutions of the original samples, a single bacterial cell grown to a small colony can be specifically identified with respect to its taxonomic and (potentially modified) genetic identity. This could be demonstrated in the present study by analysing intestinal and faecal samples from initially germ-free rats. However, in the case of conventional rats preselection of donor and/or transconjugants by using appropriate selective media such as antibiotic supplemented or CATC media was necessary. It was possible to analyse up to 2×10^3 small colonies of lactococci on membranes of 9 cm diameter. It should therefore be possible to detect one genetically modified cell among up to 2×10^3 others using a standard Petri-dish for growth. The sensitivity of the approach can be increased by performing analyses of multiple membranes in parallel. In the future, microscopic and/or luminometric analysis of microcolonies in combination with computer aided image analysis may substantially improve the resolution power of *in situ* colony hybridization. The currently most attractive hybridization technique, the *in situ* whole-cell hybridization (*DeLong* et al., 1989; *Amann* et al., 1995) which can be combined with flow cytometry depends on the presence of high numbers of target sites per cell (10^3) and therefore cannot be used for the monitoring of genetically modified organisms so far. Future developments such as *in situ* polymerase chain reaction or alternative labels of the probes may contribute to the applicability of this elegant detection method.

In general, the diagnostic PCR based detection methods for genetically modified nucleic acids are highly sensitive tools for monitoring studies. However, the sensitivities and specificities of these techniques are influenced by the chemical and organismal complexities of environmental samples. Studies on the sensitivity of the PCR approach revealed that 2×10^4 lactococci per gram represent a realistic detection limit for faecal samples. The sensitivity can be increased by dot blot hybridization of the amplified fragments to specific probes. The application of a nested PCR approach (*Schleifer* et al., in press) allows to detect 10^3 cells per gram faeces. In comparison with the *in situ* colony hybridization, a disadvantage of PCR based detection methods is, that only the presence of the target nucleic acid can be demonstrated but not its association with living cells.

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Legends to the Figures

Figure 1. Schematic representaion of the orientation and targeting of *prtP* / *prtP'* specific hybridization probes and/or amplification primers in relation to the sites of mutation.

Figure 2. Agarose gel electrophoretic separation of DNA fragments obtained by *in vitro* amplification of proteinase *prtP* gene DNA using the primer combinations PLC1 / PLC3 (lanes 1 and 2) and PLC2 / PLC3 (lines 3 and 4), respectively. Lanes 1 and 3: *Lactococcus lactis* MG1820 (pNZ511); 2 and 4: *Lactococcus lactis* MG1820 (pLMP1); S: 123 bp DNA ladder.

Figure 3. Agarose gel electrophoretic separation of DNA fragments obtained by *in vitro* amplification of proteinase *prtP* gene DNA using the primer combination PLC2 / PLC3 / PLC4. Lane 1: *Lactococcus lactis* MG1820 (pNZ511); lane 2: *Lactococcus lactis* MG1820 (pLMP1); S: 123 bp DNA ladder.

Figure 4. Mean concentrations of *Lactococcus lactis* strains in faecal samples from the rats of group A. Symbols: —, growth on rifampicin and streptomycin supplemented media; - -, growth on chloramphenicol supplemented media; No growth was detectable on chloramphenicol, rifampicin and streptomycin supplemented media. Vertical bars indicate the standard deviation.

Figure 5. Mean concentrations of *Lactococcus lactis* strains in faecal samples from the rats of group B. Symbols: —, growth on rifampicin and streptomycin supplemented media; – –, growth on erythromycin and chloramphenicol supplemented media; - -, growth on erythromycin, rifampicin and streptomycin supplemented media; No growth was detectable on chloramphenicol, erythromycin, rifampicin and streptomycin supplemented media. Vertical bars indicate the standard deviation.

Figure 6. Mean concentrations of *Lactococcus lactis* strains in faecal samples from the rats of group C. Symbols: —, growth on rifampicin and streptomycin supplemented media; – –, growth on erythromycin supplemented media; - -, growth on erythromycin, rifampicin and streptomycin supplemented media. Vertical bars indicate the standard deviation.

Table 1. Bacterial strains which were used for monitoring studies, their antibiotic resistances and plasmids.

Organism	Strain	Plasmid	Resistance	Reference
Lactococcus lactis subsp. *lactis*	MG 1820 (pNZ511)	pNZ511, pMG820	Cm	*De Vos* et al., 1989
Lactococcus lactis subsp. *lactis*	MG 1820 (pLMP1)	pLMP1, pMG820	Cm	*Hertel* et al., 1992
Lactococcus lactis subsp. *lactis*	MG 1820 (pAMβ1)	pAMβ1, pMG820	MLS	This study
Lactococcus lactis subsp. *lactis*	MG 1820 (pLMP1, pAMβ1)	pLMP1, pMG820, pAMβ1	Cm, MLS	This study
Lactococcus lactis subsp. *lactis*	MG 1363 (pLMP1)	pLMP1	Cm	This study
Lactococcus lactis subsp. *lactis*	MG 1363 (pLMP1, pAMβ1)	pLMP1, pAMβ1	MLS	This study
Lactococcus lactis subsp. *lactis* biov. *diacetylactis*	Bu-2-60	No plasmid	Rif, Str	*Neve* et al., 1984
Lactobacillus curvatus	LTH1704 (pAMβ1)	pAMβ1	MLS	*Vogel* et al., 1992

units of lactococcal strains used for dosing of three and two groups of germ-free (A, B, C) and ectively. [t] space of time between first and second dosings. Abbreviations: c.f.u., colony forming germ-free.

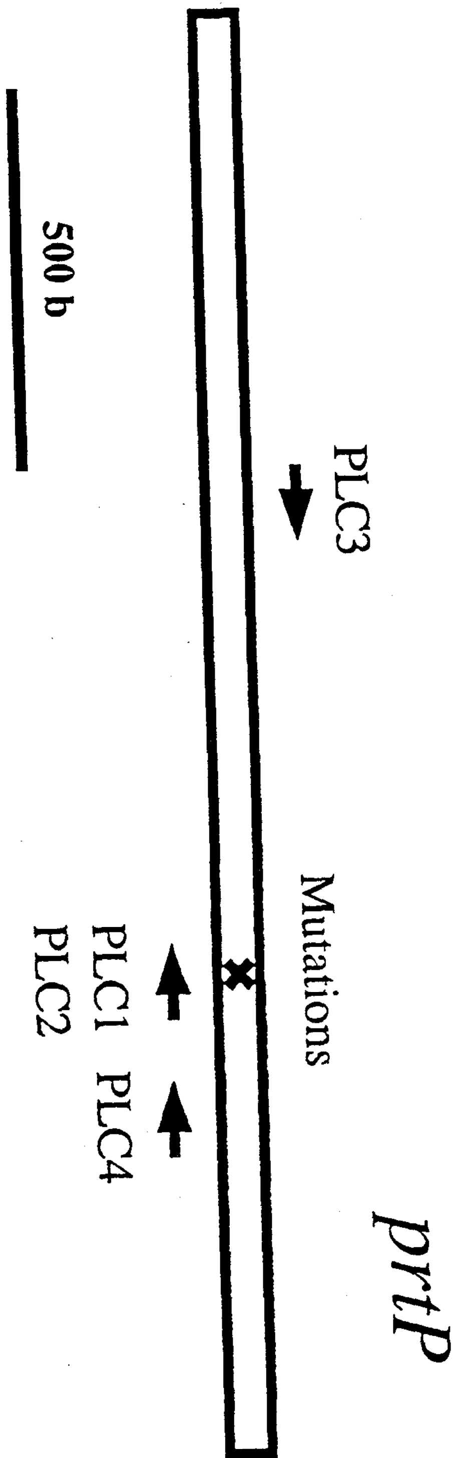
Lactococci 1st strain	c.f.u.	2nd strain	c.f.u.	Second dosage[t]
Bu2-60	3.1×10^9	MG1820 (pLMP1)	3.2×10^9	1 week
Bu2-60	3.1×10^9	MG1820 (pLMP1, pAMβ1)	3.9×10^8	2 weeks
Bu2-60	1.1×10^9	MG1820 (pAMβ1)	8.5×10^8	1 week
Bu2-60	8.2×10^8	MG1820 (pAMβ1)	1.4×10^9	15 min
Bu2-60	8.2×10^8	MG1820 (pLMP1)	1.2×10^9	15 min

ites, and specificities of primers and probes. The hybridization and washing temperatures are given in n the methods and materials section. [a] base numbering according to *Vos* et al., 1989; [b] base numbering '; [c] base numbering according to *Brosius* et al., 1981; [d] primer combination: PLC3/PLC4; [e] primer ner combination: PLC2/PLC3; [g] primer combination: PLC1/PLC3/PLC4 or PLC2/PLC3/PLC4.

	Specificity	Target	Temperature (°C)			Reference
			PCR	Probe		
			Annealing		Washing	
;TGAGTT-3	*prtP* gene	2186-2203[a]	58[e], 48[g]	42	52	*Hertel* et al., 1992
;TAAGTT-3'	*prtP* gene, modified	2186-2203[a]	50[f], 48[g]	38	46	*Hertel* et al., 1992
CATTT-3'	*prtP* gene	1598-1614[a]	50[d, f], 48[g]	—	—	This study
AGTCA-3'	*prtP* gene	2490-2506[a]	50[d], 48[g]	—	—	This study
;AACACG-3'	pAMβ1	66-83[b]	—	58	59	This study
GCATTT-3'	*Enterococcus faecalis*, 23S rRNA	[c]	—	42	47	*Betzl* et al., 1991
TCGCTAC-3'	Bacterial 23S rRNA	[c]	—	54	54	*Ludwig* et al., 1992

Table 4: Distribution of donor, recipient and transconjugant strains in the gastrointestinal system and faeces of germ-free rats at necropsy. The rats of groups A and B were necropsied after two weeks, those of group C ten days after the second dosing. The data (c.f.u. / g intestinal contents or faeces) were obtained by analyzing 5 individuals from each group. In the case of group B, the duodenum data are based on 2 ([a]), 3([b]), and 4 ([c]) individuals only. Symbols: —, below detection level.

Group	Strain	Duodenum	Jejunum	Ileum	Caecum	Colon	Faeces
A	MG1820 (pLMP1)	2.2×10^{2}	4.6×10^{4}	7.2×10^{5}	2.0×10^{7}	2.3×10^{7}	2.9×10^{6}
	BU-2-60	4.6×10^{3}	3.2×10^{4}	1.8×10^{6}	4.1×10^{8}	5.2×10^{8}	1.0×10^{9}
	BU-2-60 (pLMP1)	—	—	—	—	—	—
B	MG1820 (pLMP1, pAMβ1)	1.5×10^{2} [b]	1.7×10^{5}	2.8×10^{5}	4.4×10^{7}	2.9×10^{7}	2.9×10^{7}
	BU-2-60	1.1×10^{2} [c]	6.6×10^{5}	1.2×10^{6}	3.2×10^{8}	5.1×10^{8}	6.9×10^{8}
	BU-2-60 (pAMβ1)	3.5×10^{1} [a]	1.8×10^{4}	1.1×10^{4}	9.5×10^{3}	1.3×10^{4}	1.5×10^{4}
	BU-2-60 (pLMP1, pAMβ1)	—	—	—	—	—	—
C	MG1820 (pAMβ1)	1.1×10^{3}	3.2×10^{4}	1.2×10^{5}	3.5×10^{7}	3.3×10^{7}	4.3×10^{7}
	BU-2-60	7.8×10^{2}	9.5×10^{4}	2.8×10^{5}	8.9×10^{7}	1.0×10^{8}	1.7×10^{8}
	BU-2-60 (pAMβ1)	1.1×10^{2}	4.5×10^{3}	1.0×10^{4}	5.0×10^{4}	5.0×10^{4}	6.5×10^{4}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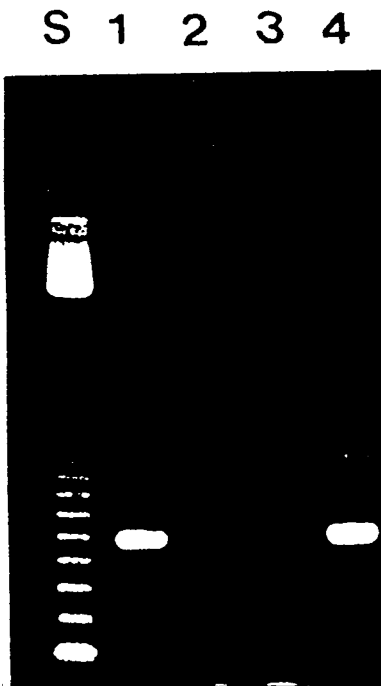
PLC3
Mutations
PLC1
PLC2
PLC4
prtP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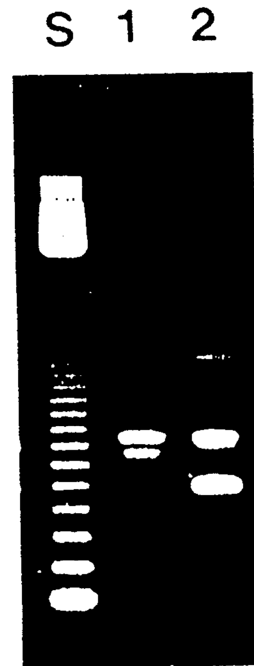
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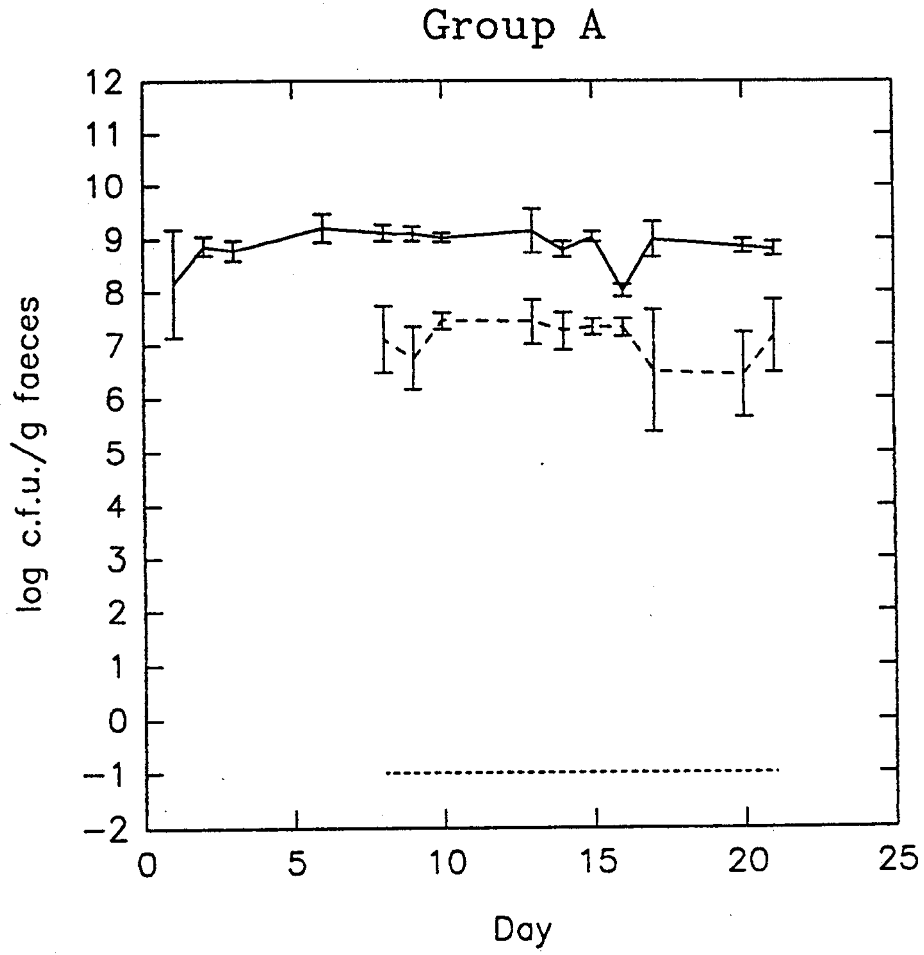
Group A
log c.f.u./g faeces
Day
12
11
10
9
8
7
6
5
4
3
2
1
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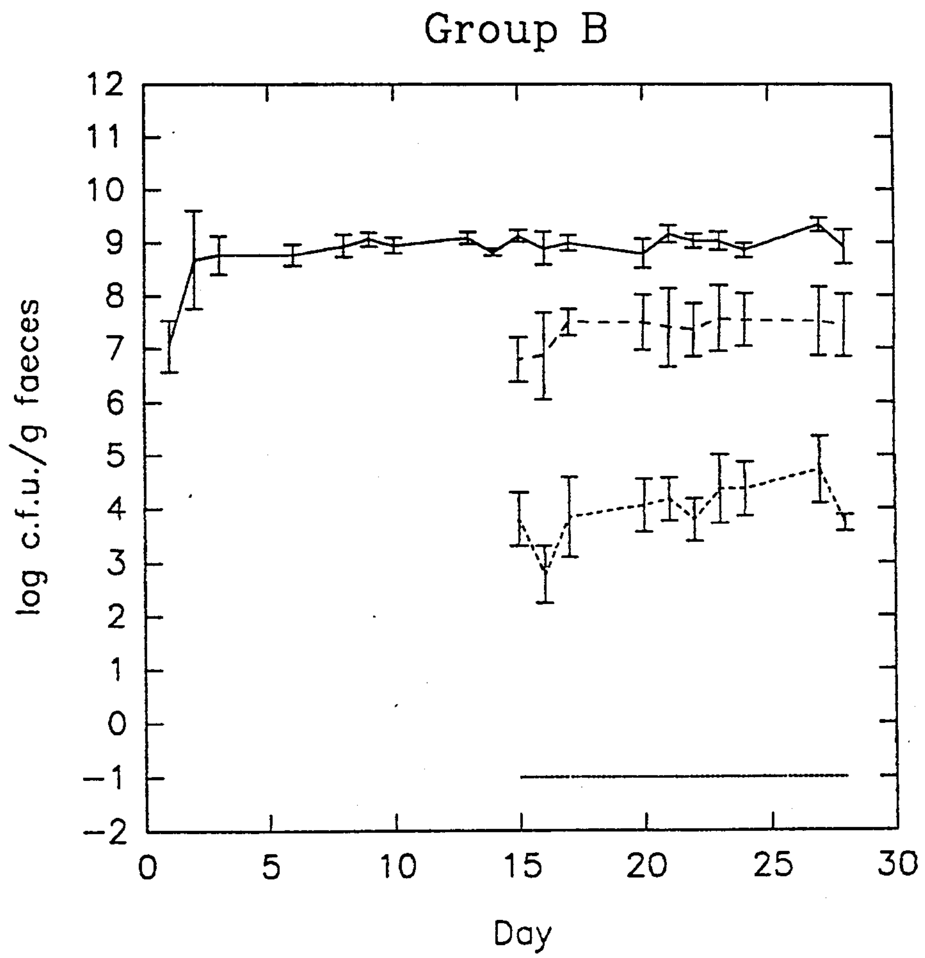
−1
−2
0
5
10
15
20
25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log c.f.u./g faeces
Day
12
11
10
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7
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4
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−2
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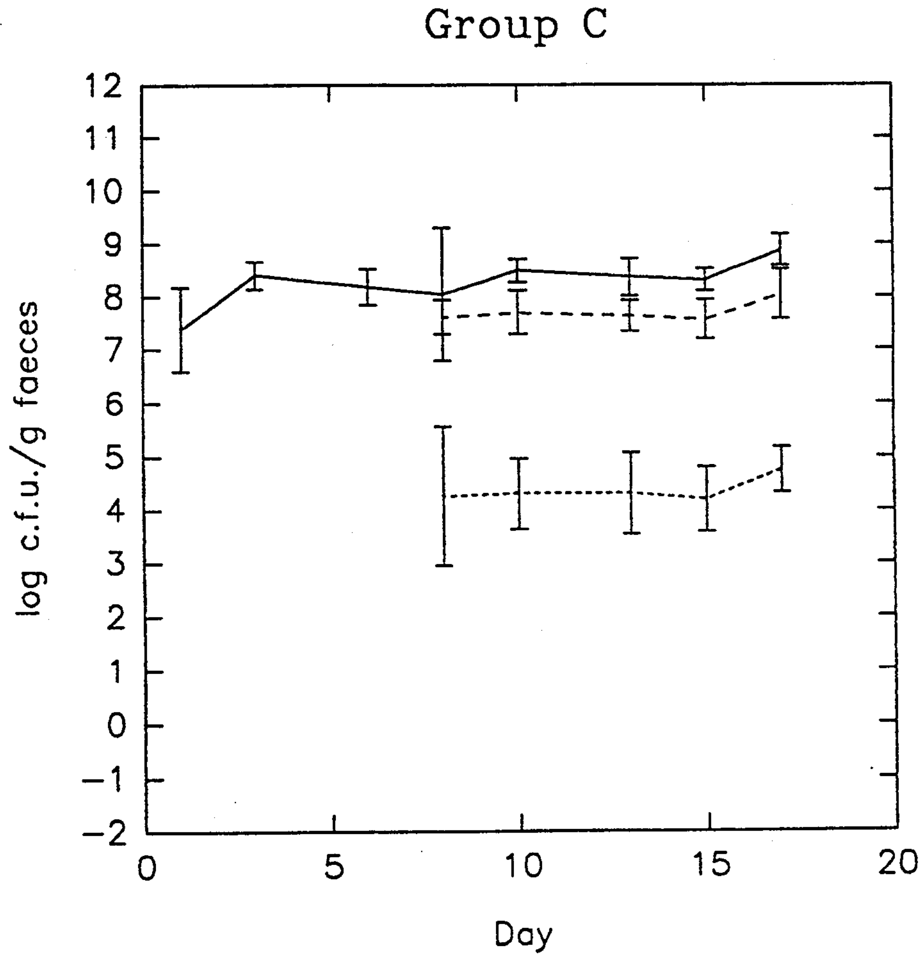
10
15
20
25
30


Group C
log c.f.u./g faeces
Day
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11
10
9
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7
6
5
4
3
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1
0
−1
−2
0
5
10
15
20