Diss. ETH No. 14676

Development of a Novel DNA Transformation System for Bifidobacteria

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
(ETHZ)

for the degree of
Doctor of Technical Sciences

presented by
Cornelia Schürch
Dipl. Lm.-Ing. ETH
born January 22, 1973
citizen of Reiden (LU)

accepted on the recommendation of
Prof. Dr. Michael Teuber, examiner
PD Dr. Hans-Martin Fischer, co-examiner
PD Dr. Leo Meile, co-examiner

Zurich, 2002

Dank

Ich möchte mich bei Herrn Prof. Dr. M. Teuber für die Vergabe dieser Dissertation bedanken.

Herrn PD Dr. L. Meile danke ich für die Betreuung und die Leitung meiner Arbeit und auch für das Vertrauen, das er mir entgegenbrachte.

Bei Herrn PD Dr. H.-M. Fischer bedanke ich mich für die Übernahme des Korreferates.

Bei den Herren Dr. A. Tauch, Dr. F. Arrigoni und E. Johansen von der Firma Chr. Hansen bedanke ich mich für die verschiedenen Stammkulturen und Vektoren, die sie mir zur Verfügung gestellt haben.

Ein herzliches Dankeschön richtet sich an alle ehemaligen und jetzigen Mitarbeiterinnen und Mitarbeiter des Labors für Lebensmittelmikrobiologie für die Hilfsbereitschaft und Offenheit und natürlich für die angenehme Arbeitsatmosphäre. Hier einschliessen möchte ich den Dank an meine Diplomanden Claudia Fretz und Matthias Hoppler und die Semestranden für Ihren engagierten Einsatz und Ihr Interesse an meiner Arbeit.

Einen besonderen Dank gebührt Melanie Stierli und Ana Alba Pérez, die mich mit Ihrer menschlichen und fachlichen Unterstützung immer wieder aufgestellt und weitergebracht haben, danke.

Frau Karin Holsten möchte ich für die Englischkorrekturen danken.

Meiner ganzen Familie und insbesondere Tobias Stutz gebührt ein ganz spezieller Dank, für Ihre Unterstützung, Ihr Glaube an mich und Ihr Verständnis während meiner gesamten Ausbildung.

Contents

Summary

Bifidobacterium strains belong to the predominant microflora of the human and animal intestine and are used as probiotic dairy product supplements. In recent years there has been an upsurge in interest in the possibility of manipulating these microorganisms genetically.

Several studies documented successful transformation experiments of *Bifidobacterium* strains with *Bifidobacterium-E. coli* shuttle vectors. Unfortunately, the *Bifidobacterium-E. coli* shuttle vectors were patent-protected, this makes them unavailable for further research. Therefore, the aims of this thesis were the establishment of an alternative transformation system for bifidobacteria. A second aim was the design of a new PCR approach to check the purity of *Bifidobacterium* cultures routinely.

In order to find a new plasmid in *Bifidobacterium* of human origin as a basic unit for transformation vectors, human faecal samples were examined for plasmid containing *Bifidobacterium* strains. All plasmids found in these bifidobacteria isolates have a size of over 8kb, which made them inconvenient for further vector constructions. Therefore, the 2.1-kb *B. asteroides* plasmid pAP1 (Kaufmann, 1998. Thesis no 12526 ETH Zürich) was used as basic unit for the construction of new *Bifidobacterium-E. coli* shuttle vectors. The plasmid pAP1 was assembled with the *E. coli* plasmid pUC18 at two different restriction sites. The two antibiotic resistance marker genes chloramphenicol-acetyltransferase (*cat*) from *Staphylococcus aureus* and the ribosome-protection tetracycline resistance determinant *tet*(W) from a *Bifidobacterium* sp. were used as selection markers. With these constituents, 7 distinct plasmids, named pCSC1 to pCSC7 had been constructed with a size of 6.9kb respectively 5.8kb.

At the end, *B. longum* NCC 2705 was transformed successfully with the 6.9-kb plasmid pCSC1 carrying the *tet*(W) gene. A transformation efficiency of 3 to 5 transformants/µg DNA was obtained which could be increased 100 fold by using reisolated DNA from a transformed *Bifidobacterium*. The plasmid structure was not rearranged and was stably maintained under selective pressure for over 20 generations.

With the successful transformation of pCSC1, it could be demonstrated that the cryptic *orf2* of pAP1 is definitively not involved in replication mechanisms, since in pCSC1 the gene was disrupted.

Furthermore, it was suggested that *B. longum* NCC 2705 does not recognise the bifidobacterial *tet*(W) promoter because pCSC2, which only differs form pCSC1 in the orientation of the *tet*(W) gene, could not transform *B. longum*.

Obtaining *Corynebacterium glutamicum* ATCC 13032 transformants with the two vectors pCSC6 and pCSC7 showed the ability of a *Bifidobacterium* plasmid to replicate in *C. glutamicum* and the functionality of the *cat* gene, which is encoded on pCSC6 and pCSC7.

The attempt failed to obtain transformed *B. longum* NCC 2705 strains expressing the green fluorescence protein gene (*gfpuv*), which is encoded on pCSC8, a descendant of pCSC1.

In this study plasmid vectors were constructed for the first time on the basis of a *B. asteroides* plasmid and the *tet*(W) gene from *Bifidobacterium* sp. was successfully used as a selection marker in bifidobacteria for the first time. These plasmid vectors could successfully transform *B. longum* or *C. glutamicum*. The transformation vectors are suitable for research purpose, but due to their low transformation rate neither ideal as standard *Bifidobacterium-E. coli* nor as *Corynebacterium-E. coli* shuttle vectors. Whether the provenience of the plasmid or another reason are crucial to the effectiveness of the vector remains unclear.

Zusammenfassung

Bifidobakterien gehören zu der vorherrschenden Mikroflora des menschlichen und tierischen Intestinaltraktes. Da Bifidobakterien in Milchprodukten als probiotische Zusätze eingesetzt werden, wuchs das Interesse der Industrie an diesen anaeroben Bakterien und ihrer genetischen Manipulation mit der aufkeimenden Propagierung von gesundheitsfördernden Lebensmitteln.

In verschiedenen Studien wurden daraufhin erfolgreiche Transformationen von *Bifidobacterium* Stämmen mit *Bifidobacterium-E. coli* Shuttlevektoren beschrieben. Leider sind diese patentgeschützten *Bifidobacterium-E. coli* Vektoren nicht für weitere Forschungszwecke erhältlich. Deshalb war das Ziel dieser Arbeit, ein neues Transformationssystem für Bifidobakterien zu etablieren und zusätzlich eine neue PCR Strategie zur Routinekontrolle von *Bifidobacterium* Kulturen zu entwickeln.

Als Grundlage für neue Transformationsvektoren sollte ein Plasmid aus einem *Bifidobacterium* menschlichen Ursprungs verwendet werden, deshalb wurden plasmidhaltige Bifidobakterien in menschlichen Stuhlproben gesucht. Alle gefundenen Plasmide waren grösser als 8kb, was weitere Vektorkonstruktionen erschwerte. Deshalb wurde das 2.1kb-Plasmids von *B. asteroides* (Kaufmann, 1998. Thesis no 12526 ETH Zürich) als Ursprung für die neuen Vektoren gewählt. Das Plasmid pAP1 wurde mit dem *E. coli* Plasmid pUC18 an zwei verschiedenen Restriktionsstellen ligiert und als Selektionsmarker wurden die Gene der Chloramphenicol-Acetyltransferase (*cat*) aus *Staphylococcus aureus* und der ribosomal geschützten Tetracyclinresistenz *tet*(W) einer *Bifidobacterium* sp. verwendet. Mit diesen Bestandteilen wurden die 7 Vektorplasmide pCSC1 bis pCSC7 konstruiert, die eine Grösse von 6.9kb, beziehungsweise 5.8kb aufwiesen.

Der Stamm *B. longum* NCC 2705 konnte nur mit dem 6.9kb Plasmid pCSC1, welches das *tet*(W) Gen beinhaltet, erfolgreich transformiert werden. Die Transformationseffizienz betrug 3-5 Transformanten/µg DNA, konnte aber hundertfach gesteigert werden, wenn Plasmid DNA aus transformierten Bifidobakterien, anstatt aus *E. coli* verwendet wurde. Unter Selektionsdruck war das Plasmid während über 20 Generationen ohne Strukturveränderungen stabil.

Mit der Transformation von pCSC1 konnte bewiesen werden, dass die kryptische *orf2* Region von pAP1 nicht an der Replikation des Plasmides beteiligt ist, da in Plasmid pCSC1 dieser *orf2,* bedingt durch die Vektorkonstruktion, zerstört war. Zusätzlich wird vermutet, dass *B. longum* NCC 2705 den *tet*(W) eigenen bifidobakterien Promoter nicht erkennt, da das Plasmid pCSC2, das sich nur durch die Orientierung des *tet*(W) Gens von pCSC1 unterscheidet, Bifidobakterien nicht transformieren konnte.

Die erfolgreiche Transformation von *Corynebacterium glutamicum* ATCC 13032 mit den Plasmiden pCSC6 und pCSC7 zeigte, dass das Bifidobakterien Replikon von pAP1 in den Vektoren nicht nur in Bifidobakterien sondern auch in Corynebakterien funktioniert und dass *C. glutamicum* das *cat* Gen exprimieren kann.

Der Versuch in *B. longum* NCC 2705 das in Plasmid pCSC1 integrierte grünfluoreszierende Gen *gfpuv* zu exprimieren, schlug fehl.

In dieser Arbeit wurde zum ersten Mal ein Plasmidvektor, der auf einem *B. asteroides* Plasmid beruht, erfolgreich sowohl in Bifidobakterien als auch in Corynebakterien transformiert. Als Selektionsmarker konnte ebenfalls zum ersten Mal das *tet*(W) Gen in Bifidobakterien eingesetzt werden. Durch die geringen Transformationseffizienzen sind die neu entwickelten Vektoren zwar für Forschungszwecke geeignet, aber weder als *Bifidobacterium-E. coli* noch als *Corynebacterium-E. coli* Shuttlevektoren einsetzbar. Ob die Herkunft der Ursprungsplasmide oder andere Gründe im Hinblick auf die geringe Effektivität der Vektoren eine Rolle spielen bleibt unklar.

1 Introduction

1.1 The characteristics of the genus *Bifidobacterium*

Bifidobacteria are Gram-positive, anaerobic, non-motile, non-sporeforming, catalase-negative, fermentative rods of various shapes, usually curved and clubbed and are often Y- or V- branched.

Theoretically, bifidobacteria actively ferment carbohydrates mainly producing acetic and lactic acids in the molar ratio of 3:2 without the production of CO_2. However, the study of Meile *et al.* (1997) showed that often lactic acid production is replaced by formic acid production (up to 24mM). In these cases, the molar ratio of acetic to lactic acid is 10:1. Glucose is exclusively and characteristically degraded by the fructose 6-phosphate shunt with the key enzyme fructose 6-phosphate phosphoketolase. The encoding gene of the fructose 6-phosphate phosphoketolase was cloned very recently (Meile *et al.*, 2001). Neither aldolase nor glucose-6-phosphate dehydrogenase activity are detectable in protein extracts of *Bifidobacterium*.

The habitat of bifidobacteria is the intestinal tract and oral cavity of warm-blooded vertebrates, the gut of insects, and sewage. They are said to be involved in certain human infective processes (mainly dental caries) but usually are considered non-pathogenic. The G+C content of the DNA varies from 55 to 67mol% (Scardovi, 1986), only *B. inopinatum* has a lower G+C content of 45mol% (Crociani *et al*., 1996).

1.2 A short history of *Bifidobacterium*

Bifidobacteria were first described by Tissier 1900 at the Institute Pasteur. He found these rodshaped, anaerobic bacteria as predominant flora in the faeces of breast-fed infants and named them *Bacillus bifidus communis* in respect of their Y-shaped morphology (lat. *bifidus*: cleft, divided).

Based on morphological, cultural and biochemical characteristics, Orla-Jensen suggested in 1924 to classify these bacteria in the genus *Bifidobacterium* with the type species *Bifidobacterium bifidum*. Even though, in the VIIth edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957) the name *Lactobacillus bifidus* was still used for bifidobacteria. Mainly three different properties of bifidobacteria led to their final separation and reclassification as a separate genus in the VIIIth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974): sugar fermentation (Dehnert, 1957; Reuter, 1963), the existence of the fructose 6-phosphate phosphoketolase (De Vries and Stouthamer, 1967) and the high G+C content of the bifidobacterial DNA.

1.3 Advances in the taxonomy of *Bifidobacterium*

The taxonomy of bifidobacteria has continuously changed since they were first isolated. Initially, the differentiation of bifidobacteria species was based on physiological and morphological traits like carbohydrate fermentation patterns, cell wall and cellular fatty acids composition or serological characteristics. Afterwards, DNA-DNA filter hybridisation procedures were used to recognise new DNA homology groups among bifidobacterial strains. With the use of polyacrylamide gel electrophoresis of soluble cellular proteins bifidobacteria species were distinguished by polyamine and whole-cell protein analysis using SDS-PAGE (Biavati *et al*., 1992).

Nevertheless, the discrimination on species level was problematic as the carbohydrate fermentation leads to ambiguous results and in several cases even fails. Only the progress in molecular genetics opened completely new views into taxonomy. The modern taxonomic tools were based on genotype studies such as the mol% G+C composition of the DNA, DNA-DNA hybridisation (Biavati and Mattarelli, 1991) and hybridisation with specific DNA probes (Langendijk *et al.*, 1995; Tannock *et al.*, 1996; Kaufmann *et al.*, 1997b). The use of genotyping methods like pulsed-field gel electrophoresis (Bourget *et al.*, 1993; Roy *et al.*, 1996), rRNA gene restriction patterns (Mangin *et al.*, 1999) and several methods using polymerase chain reactions with specific rDNA or rRNA probes are extended approaches for taxonomy (Weisburg *et al.*, 1991; Matsuki *et al.*, 1998; Brigidi *et al.*, 2000; Ventura *et al.*, 2001).

Nowadays most of taxonomic or also phylogenetic studies were based on highly conserved rDNA or rRNA regions, above all the 16S rRNA and rDNA, the 23S rRNA and the 16S-23S rRNA internal transcribed spacer region (Miyake *et al.*, 1998). Dependent on the chosen target of these regions, a separation on genus, species or even strain level is possible.

The combination of all these above mentioned phenotypic and genotypic characteristics led to a new polyphasic approach in taxonomy (Vandamme *et al.*, 1996).

The group of Jian recently published a new approach to phylogenetically analyse the genus *Bifidobacterium*, based on sequence homologies of a portion of *hsp60* genes encoding the 60-kDa heat shock proteins (Jian *et al.*, 2001). According to the authors, phylogenetic tree calculations were basically similar to those of the 16S rRNA, but the tree seemed to be more clear-cut for species delineation and the clustering was better correlated with the mol% G+C content of the species and subspecies.

However, so far, the genus *Bifidobacterium* is classified within the domain *Bacteria* into the class *Actinobacteria* (Stackebrandt *et al.*, 1997) within the subclass *Actinobacteridae*, the order *Bifidobacteriales* and the family *Bifidobacteriaceae* including also the genus *Gardnerella*. According to former taxonomic criteria, bifidobacteria were phylogenetically grouped in the actinomycete branch of Gram-positive bacteria within the high G+C cluster (Olsen *et al*., 1994).

On the basis of DNA-DNA homology, 33 different species of bifidobacteria are currently existing: 11 human species, 15 species of warm blooded animals, 3 species of honeybees, 3 species isolated from waste water and 1 species from fermented milk (Watabe *et al.*, 1983; Scardovi, 1986; Lauer, 1990; Biavati and Mattarelli, 1991; Biavati *et al.*, 1991; Crociani *et al.*, 1996; Meile *et al.*, 1997), including the only recently discovered species *B. thermacidophilum* (Dong *et al.*, 2000).

Nevertheless, the separation of bifidobacteria from lactobacilli for direct identification and enumeration in dairy foods like yoghurt and cheese or to monitor bifidobacteria as indicator for faecal contamination (Nebra and Blanch, 1999) is still difficult. No truly selective medium exists, although a lot of efforts has been made in finding selective media for bifidobacteria (Charteris *et al.*, 1997; Rada and Koc, 2000). In selective media described in different studies, most effective supplements for bifidobacteria selection were antibiotics like neomycin, paramomycin, nalidixic acid (Tanaka and Mutai, 1980) and mupirocin (Nebra and Blanch, 1999) or supplements like propionic acid (Beerens, 1990) or lithium chloride (Hartemink *et al.*, 1996). However, none of these media turned out to be truly selective for bifidobacteria.

Based upon modern taxonomic tools further methods to identify and enumerate bifidobacteria in various samples were developed. A few examples are the use of box primers for PCR (Gómez Zavaglia *et al.*, 2000), or to use PCR methods combined with ribotyping methods such as amplified rDNA restriction analysis (Roy and Sirois, 2000) and specific PCR with denaturing or temperature gradient gel electrophoresis (Akkermans *et al.*, 1998; Satokari *et al.*, 2001).

1.4 *Bifidobacterium* and their role as food supplement

1.4.1 Probiotics

The idea to use viable bacteria as food supplement is not new, at the beginning of the last century first scientific work had been done by Metchnikoff to investigate the beneficial effects of fermented milk for human health (Metchnikoff, 1907). Since then several studies have been made on this subject until finally Fuller tried to define probiotics as "a live microbial feed supplement that beneficially affects the host (animal) by improving its intestinal microbial balance" (Fuller, 1989).

Controversial discussion of the following claims of probiotics dominate among the scientific community: Upon daily intake, probiotic bacteria are said to colonise the gastrointestinal tract and exert their beneficial effects on the host by (i) maintaining a healthy microflora (prevention of diarrhoea), (ii) inhibiting the growth of pathogenic bacteria by producing bacteriocins and lowering the pH value by acetate and lactate production, (iii) improving lactose tolerance, (iv) reducing serum cholesterol levels, (v) reducing blood ammonia concentration, (vi) producing vitamins, e.g. folate (Deguchi *et al.*, 1985)), (vii) stimulating the immune system, and (viii) having an anticarcinogenic and antimutagenic activity (Mitsuoka, 1990; Ballongue, 1993).

The fact that the mechanisms have not been identified by which probiotic microorganisms antagonise unwanted gastrointestinal tract microorganisms or exert other beneficial effects to the host *in vivo*, and that not all *in vivo* studies are random, double-blind, placebo controlled and are based on more than 50 volunteers prevents the scientific acceptance of the promotional health claims (Atlas, 1999).

The probiotic concept itself is highly controversial since it neglects the colonising resistance of newly introduced "non-autochthonous" bacteria into the human intestine (Meile, 1998). Thus so far, for example in the studies of Kleessen *et al.* (1997) and Kruse *et al.* (1999), no permanent colonisation of the intestine by consumed bifidobacteria could be detected after stopping continuous intake. To substantiate all the predicted beneficial effects, further research is urgently needed (Teuber, 2000).
Tannock (1999) wrote an excellent review about this topic with more detailed information.

1.4.2 *Bifidobacterium* and their role as probiotics

The most common bacteria cultures used as probiotic food additives with GRAS (Generally Recognised As Safe) status are *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* sp.
These strains were selected, because they were simple to cultivate outside the intestine, and additionally, lactobacilli were classical production strains for fermented milk products. The use of bifidobacteria as probiotics is to ascribe to their normal habitat in the intestine throughout the whole life cycle as predominant flora (Tannock *et al*., 1996). The important role of bifidobacteria in the gastrointestinal tract microflora was already described by Tissier. Nowadays the composition of the gut microflora is better studied and is determined routinely by *in situ* hybridisation with specific 16S rRNA probes (Meile, 1998; Kleessen *et al.*, 2001; Rycroft *et al.*, 2001; Arrigoni *et al.*, 2002). This high accurate method revealed that within the 10^{10}-10^{11} bacteria per gram found in the colon bifidobacteria constitute between 5-15%. Beside *Bifidobacterium*, also *Bacteroides* and *Eubacteria* belong to the predominant species of a "normal" microbial ecosystem which comprises more than 500 different bacterial species (Salminen *et al.*, 1995, Tannock, 1999). This gastrointestinal tract flora is a host- and location-specific complex and a strict-anaerobic ecosystem. It is generally assumed that the composition and activity of this microflora influences the host's health, and exerts strong competition against any organism that might be introduced into the balanced microbial community (Berg, 1998).
Furthermore, it was supposed that bifidobacteria as normal and predominant inhabitants of the human intestinal tract throughout the life cycle are the most important bacteria for health promoting effects (Mitsuoka, 1990; Ballongue, 1993). But sufficient scientific data does not exist to substantiate this.

In addition to this, the group of Heinig found that breast-fed infants harbour higher numbers of *Bifidobacterium* and are less at risk for diarrhoeal diseases or susceptible to infections than bottle-fed children (Heinig and Dewey, 1996).
All these aspects eventually led to the increased interest and importance of the genus *Bifidobacterium* for the industry as probiotic food supplement.

1.5 Molecular genetics of *Bifidobacterium*

Since bifidobacteria are considered to exert probiotic effects and were used therefore as food supplements, the industrial interest to find new, suitable production strains have risen. Thus, progresses in applied genetics of bifidobacteria has been needed and in consequence, many studies were made in this research field. On one hand, new bifidobacteria strains were isolated and characterised. On the other hand, several attempts were made to genetically modify well known strains by recombinant DNA techniques to obtain industrial strains with all the properties the industry requires.
However, for both aims, the characterisation of industrial strains as well as the genetically modification of bifidobacteria, the screening of *Bifidobacterium* strains for plasmid occurrence is the first thing to do. After that, the genetic properties of bifidobacteria have to be determined, relevant genes have to be found, sequenced, and their functions have to be identified. With these basic elements, an efficient transformation system for bifidobacteria should be developed for use in recombinant DNA technologies and for strain improvement.

1.5.1 Plasmids in *Bifidobacterium*

Crucial to the genetic engineering of the genus *Bifidobacterium* is the availability of small molecular size cloning vectors which are structurally stable and allow efficient cloning and maintenance of heterologous DNA fragments. The most important feature of a potential vector is the ability to replicate in bifidobacteria. There are three possibilities: (i) The vector is based on a replicon derived from *Bifidobacterium*; (ii) plasmids are used which derive from bacteria closely related to *Bifidobacterium*; (iii) broad host range plasmids that replicate in a wide variety of Gram-positive and Gram-negative bacteria are also suitable in bifidobacteria.

The most effective possibility could be the first one, to use a replicon derived from *Bifidobacterium* itself. Therefore, the search for plasmids in bifidobacteria is an important first step. Plasmids were described in six different species*: B. breve, B. pseudocatenulatum* and *B. longum* from the human intestinal tract, *B. asteroides* and *B. indicum* isolated from honeybees and *B. globosum* found in various animals (Sgorbati *et al.*, 1982; Sgorbati *et al.*, 1986a; Sgorbati *et al.*, 1986b; Iwata and Morishita, 1989; Matteuzzi *et al.*, 1990; Bourget *et al.*, 1993; Mattarelli *et al.*, 1994; Park *et al.*, 1997; Smeianov *et al.*, 2002).

These species normally harbour multiple plasmids, in sizes of 1.25 to 9.5kb. Most of these plasmids are cryptic and have not been characterised beyond restriction mapping. Six plasmids have been completely sequenced: pMB1 from *B. longum* (Rossi *et al.*, 1996), pAP1 from *B. asteroides* (Kaufmann *et al.*, 1997a), pCIBb1 from *B. breve* (O'Riordan and Fitzgerald, 1999), pKJ36 and pKJ50 from *B. longum* (Park *et al.*, 1997) and p4M from *B. pseudocatenulatum* (Smeianov *et al.*, 2002).

1.5.2 Transformation of *Bifidobacterium*

Several *Bifidobacterium-E. coli* shuttle vectors have been constructed. They are of the general utility type with distinct replication origins and markers selectable in bifidobacteria and *E. coli.* Most of these vectors derive from the small cryptic plasmid pMB1, for example the shuttle vector pRM2 (Missich *et al.*, 1994) used to transform bifidobacteria for the first time, the vector pNC7 (Rossi *et al.*, 1997) or the vector pDG7 (Argnani *et al.*, 1996). Two vectors have been constructed from other, less well-defined plasmids. These are pBLES100 derived from pTB6 (Matsumura *et al.*, 1997) and the *Bifidobacterium-E. coli* shuttle vector constructed from plasmid pKJ36 (Park *et al.*, 2000). The antibiotic resistance determinants for selection in these vectors are for chloramphenicol, spectinomycin or erythromycin in bifidobacteria and for ampicillin in *E. coli*.

In 1990, the group of Matteuzzi *et al.* (1990) constructed two different vectors: pDG7 and pMR3 consisting of pMB1, isolated from *B. longum* (Sgorbati *et al.*, 1982) and cloned into two different cloning sites of *E. coli* vector pJH101 (Ferrari *et al.*, 1983). They tried in vain to transform *Bacillus subtilis* and *Lactobacillus reuteri* with these vectors.

In 1994, the group of Missich *et al.* (1994) successfully introduced the vector pRM2 by electrotransformation into the cured *B. longum* strain which originally harboured plasmid pMB1.

Plasmid pRM2 was constructed by assembling pMB1 with a commercially available *E. coli* transformation vector, containing a spectinomycin resistance gene. They used the *E. coli* transformation protocol and yielded very low efficiencies of $3.8x10^2$ transformants/μg DNA.

The group of Matsumura used other *B. longum* strains for transformation with vector pEBLES100 consisting of the *B. longum* plasmid pRB6, pBR322 from *E. coli* and a spectinomycin resistance gene (Matsumura *et al.*, 1997). They also used the transformation protocol from Missich *et al.* (1994) and obtained slightly better efficiencies of $2.2x10^4$ transformants/μg DNA.

At about the same time, the group of Argnani *et al.* (1996) used the already mentioned pDG7 vector of Matteuzzi *et al.* (1990) to transform different bifidobacteria strains including *B. animalis, B. longum, B. bifidum, B. breve,* and *B. infantis* with a new, more adapted transformation protocol which produced transformation efficiencies ranging from $7.2x10^3$ to $7x10^4$ cfu/μg DNA. Furthermore, Argnani transformed successfully all above mentioned bifidobacteria with two different *Corynebacterium-E. coli* shuttle vectors but obtained a lower efficiency of $2x10^3$ transformants/μg DNA. Nevertheless, she showed that a *Corynebacterium* replicon also works in bifidobacteria (Argnani *et al.*, 1996).

The group of Rossi used various vectors based on pMB1 which was cloned into the commercial *E. coli* vector pBluescript. Rossi tested an even wider range of bifidobacterial host strains with *B. animalis* as the most important strain and used the transformation protocol of Argnani with efficiencies of $3.7x10^3$ transformants/μg DNA (Rossi *et al.*, 1996). With a better adapted method an efficiency of $3x10^4$ cfu/μg DNA was achieved by using vector DNA isolated not from *E. coli* but from a bifidobacterial transformant (Rossi *et al.*, 1997). The most recent transformation experiment with a *B. longum* strain was reported by the group of Park and yielded in turn again a low efficiency of $2.6x10^2$ cfu/μg DNA (Park *et al.*, 2000).

All transformation experiments with *Bifidobacterium* strains reported so far are summarised in table 1-1.

Table 1-1: Summary of all transformation experiments with *Bifidobacterium*.

Host strain	Plasmid	Properties[a]	Transformants/ µg DNA[b]	DNA amount[c]	Reference
B. longum	pRM1 4.9kb	pMB1 + pGEM-5Zf(+)	nt	0.5-1µg	Missich *et al.*
	pRM2 6.0kb	pRM1 + $Spec^R$	$3.8x10^2$		(1994)
B. animalis	pDG7 7.3kb	pMB1 + pBR322+ Cm^R	$5x10^3$	0.5-1.5µg	Argnani *et al.*
B. bifidum			$7.4x10^3$		(1996)
B. infantis			$4x10^4$		
B. breve			$2x10^2$-$1.3x10^4$		
B. longum			$2.6x10^3$-$7x10^4$		
B. animalis	pGK12 4.4kb	Ery^R + Cm^R broad host	nto	0.5-1.5µg	Argnani *et al.*
	pLP825 7.3kb	Ap^R + Cm^R	nto		(1996)
	pEBM3 9.6kb	Km^R + Cm^R	$2x10^3$		
	pECM2 10.3kb	Km^R + Cm^R	$2x10^3$		
B. animalis	pDH7	pDG7 - *orf2* of pMB1	nto	nk	Rossi *et al.*
	pKG7	pDG7 - 200bp	+		(1996)
	pNC7	pDG7 - Ap^R - *E. coli rep*	+		
	pDGE7	pDG7 + Ery^R	+		
B. longum	pNC7* 4.9kb	pDG7 - Ap^R - *E. coli rep*	$4.1x10^2$	0.25µg	Rossi *et al.*
B. bifidum			$7.2x10^4$		(1997)
B. breve			$2.3x10^4$		
B. infantis			$1.2x10^3$		
B. animalis			$3x10^4$		
B. pseudocatenulatum			$5x10^1$		
B. ruminale			$7.2x10^2$		
B. dentium			$3.6x10^1$		
B. magnum			$1.8x10^3$		
B. longum	pBLES100 9.1kb	pTB6 + pBRR322 + $Spec^R$	$2.6x10^3$-$1.6x10^4$	nk	Matsumura *et al.* (1997)
B. animalis	pDGA7 10.1kb	pDG7 + α-amylase	+	nk	Rossi *et al.*
	pDCO7 10.9kb	pDG7 + cholesterol oxidase operon	nto**		(1998)
	pDLI41 11.3kb	pDG7 + lipase	+		
	pSPEC1 5.9kb	pMB1 + $Spec^R$ + pBluescript KS	+		
	pCLJ15 5.9kb	pMB1 + Ery^R + pBluescript KS	+		
	pLF5 5.7kb	pMB1 + Cm^R + pBluescriptKS	+		
	pTRE3 2.8kb	pLF5 – pBluescript KS	+		
B. infantis	pDCO7 10.9kb	pDG7 + cholesterol	+	nk	Rossi *et al.*
B. bifidum		oxidase operon	nto**		(1998)
B. longum			nto**		
B. magnum			+		
B. infantis	pDGA7 10.1kb	pDG7 + α-amylase	+ (all plasmids)	nk	Rossi *et al.*
B. bifidum	pDLI41 11.3kb	pDG7 + lipase	+ (all plasmids)		(1998)
B. longum	pSPEC1 5.9kb	pMB1 + $Spec^R$ +	+ (all plasmids)		
B. magnum		pBluescript KS	+ (all plasmids)		
	pCLJ15 5.9kb	pMB1 + Ery^R + pBluescript KS			
	pLF5 5.7kb	pMB1 + Cm^R + pBluescript KS			
B. longum		pKJ36 + pBR322 + Cm^R	$1.2x10^1$-$2.6x10^2$	nk	Park *et al.* (2000)
		pKJ50 + pBR322 + Cm^R			

[a] Ap^R: ampicillin resistance; Cm^R: chloramphenicol resistance; $Spec^R$: spectinomycin resistance; Ery^R: erythromycin resistance; - deleted fragment

[b] nt: not tested, nto: no transformants obtained; nto**: transformation successful with DNA isolated from *Bifidobacterium* strains; +: successful transformation

[c] DNA amount used for transformation; nk: not known

* DNA isolated from *Bifidobacterium* strains

All these transformation experiments aimed at a convenient vector suitable for genetic engineering of *Bifidobacterium* species to investigate and improve their characteristics. Although these vectors seem to work, their eventual use in the food industry is questionable because the food-grade status is not given due to their antibiotic selection marker. Therefore, further research is needed to find suitable genes to be used as selection markers in a future food-grade vector.

Electrotransformation is the simplest and most convenient method at the moment to transform not naturally competent bacteria (Steele *et al.*, 1994). Several factors seem to be responsible for the low efficiencies. First of all, the thick cell wall in Gram-positive bacteria generally is a barrier for the uptake of exogenous DNA. Another difficulty is the toxicity of oxygen for the anaerobic bifidobacteria which complicates handling. Furthermore, the applied transformation protocols had been optimised for bacteria other than bifidobacteria. Only the protocol of Rossi *et al*. (1997) and Argnani *et al*. (1996) employed improved electroporation conditions for bifidobacteria resulting in slightly higher efficiencies. To obtain a reproducible, high efficient transformation rate, a protocol especially optimised for *Bifidobacterium* species is urgently needed.

1.5.3 Characterisation of genes from *Bifidobacterium*

The possibility to manipulate industrial bifidobacteria strains genetically is very promising, although so far no food-grade vector is available. The characterisation of new genes of bifidobacteria could increase the knowledge of their genetics, leading to the ability to genetically modify wild type strains. Moreover, a homologous gene might be used as food-grade selection marker in bifidobacterial vectors.

Several studies about bifidobacteria genomics has been made so far. For example, Bourget *et al*. (1993) estimated the genome size of five *B. breve* strains by pulsed-field gel electrophoresis at about 2.1Mb. Also other genomes of industrial relevant *Bifidobacterium* strains were examined and sequenced, but these data are not accessible.

Only few complete *Bifidobacterium* genes have been characterised and analysed regarding their structure, organisation, expression and regulation. Although many 16S rDNA sequences of *Bifidobacterium* species are known, only 35 complete sequences of genes of bifidobacteria are available at the moment in the GenEMBL database as shown in table 1-2.

Table 1-2: List of all complete bifidobacteria genes available in the GenEMBL database.

Genes or Protein:	Organism	Acc. Number
1. β-galactosidase (*lacZ*), *sbp*	*B. adolescentis*	AF213175
2. β-galactosidase (*lacZ*)	*B. breve*	E05040
	B. breve	BBD311
	B. bifidum	BBI272131
	B. bifidum	BBJ224434
	B. bifidum	BBJ224435
	B. bifidum	BIJ224436
	B. longum	BL0242596
	B. infantis	AF192265
	B. infantis	AF192266
3. *lacZ, tRNA-Pro*, *tag* genes	*B. longum*	BLO0242596
4. α-galactosidase, *nhaB*, cytosine deaminase	*B. longum*	AF160969
5. α-galactosidase	*B. adolescentis*	AF124596
	B. breve	AF406640
6. catabolite control protein (*ccp*), α-glucosidase	*B. adolescentis*	AF411186
7. L-lactat-dehydrogenase (*ldh*)	*B. longum*	BLLLD
8. transaldolase	*B. infantis*	AF417540
9. bile-salt-hydrolase	*B. longum*	AF148138
10. IS-element	*B. lactis*	BLA243948
11. phosphoketolase (*xfp*)	*B. lactis*	BLA293946
12. *recA*	*B. breve*	AF094756
13. replication protein	*B. breve*	E17316
14. heat shock protein 60 (*hsp60*)	*B. adolescentis*	AF210319
	B. denticolens	AF240565
	B. inopinatum	AY004281
	B. animalis	AY004282
15. *hup*, histone-like HU protein HB1	*B. longum*	AB072446
16. *tet*(W)	*B.* sp.	AF202986

14 genes encode for α- or β-galactosidases. These genes could be useful as a selection marker like *lacZ* from *E. coli* which allows the blue/white screening of cloned strains. Such an enzyme as selection marker would certainly be accepted in a food-grade vector. Unfortunately, the use of a *lacZ* gene as selection marker in *Bifidobacterium* is not simple, because it is known that at least 3 different *lacZ* genes are present in the genome of bifidobacteria (Rossi *et al.*, 2000a; Meile and F. Arrigoni, personal communication).

1.5.3.1 The *tet*(W) gene from *Bifidobacterium*

For this study the ribosome-protection tetracycline resistance determinant gene *tet*(W), listed in table 1-2, plays an important role as selection marker in the vector system to be constructed. It has been isolated from a *Bifidobacterium* strain and its sequence has been determined by Jerusalem *et al*. (2001).

The first *tet*(W) had been found in a rumen isolate of *Butyrivibrio fibrisolvens* (GenBank acc. no. AJ222769) (Barbosa *et al.*, 1999). A sequence comparison of these two *tet*(W) genes revealed 98.3% identity. The group of Scott *et al.* (2000a) found further *tet*(W) genes in the anaerobe human faeces bacteria *Fusobacterium prausnitzii* and *Bifidobacterium longum* with a 99.9% sequence identity to *Butyrivibrio fibrisolvens tet*(W). They concluded that the high level of sequence identity between the *tet*(W) genes found in bacteria of different genera and isolated from different hosts implies recent horizontal gene transfer events. Furthermore, the *tet*(W) gene with its high G+C content (53mol%) generally seems to be associated with high G+C content bacteria. All these facts led to the conviction that *tet*(W) could be suitable as selection marker for a transformation system for bifidobacteria.

1.5.4 The modified green fluorescent protein GFPuv as reporter marker

An alternative to the use of a resistance gene as selection marker is to use the broad host strain reporter gene encoding the intrinsically green fluorescencing protein from the jellyfish *Aequorea victoria*.

This gene has many advantages as it requires no exogenous substrates or cofactors, no sample preparation and is compatible with many fixation techniques. The protein is stable in a wide pH range from 7 to 11, survives heat treatment up to 65°C and is resistant to denaturants and proteases (Aspiras *et al.*, 2000). With these properties it is possible to detect gene expression, to localise proteins or to visualise the cell morphology of living cells. In addition, protein fusions to GFP can be used to screen mutants rapidly or to observe dynamic processes, such as chromosome segregational (Webb and Resnekov, 1999).

As a reporter and marker gene, *gfp* is broadly applicable in use with both prokaryotic and eukaryotic cells like *E. coli, Bacillus subtilis* (Feucht and Lewis, 2001; Itaya *et al.*, 2001), lactic acid bacteria (Scott *et al.*, 2000b) or strains of streptococci (Aspiras *et al.*, 2000).

Scott *et al.* (2000b) illustrated the functionality of the GFP as marker protein also under anaerobic conditions. Although the protein requires exposure to oxygen for fluorescence, plating in air with subsequent anaerobic incubation was sufficient to form the chromophor (Scott *et al.*, 2000b). Meanwhile, the *gfp* has been modified and several vectors containing *gfp* variants are commercially available. These *gfp* vectors has also been developed for specific use in eukaryotic cells.

1.6 The aim of this work

Since all the above mentioned *Bifidobacterium-E. coli* shuttle vectors are patent protected and thus not available, a new shuttle vector was needed for transformation experiments. Therefore, the aim of this thesis was the development of *Bifidobacterium-E. coli* shuttle vectors based on *E. coli* plasmid pUC18 and either on newly found plasmids of bifidobacteria isolated from human faeces, or on the well-characterised *Bifidobacterium asteroides* plasmid pAP1.

2 Materials and Methods

2.1 Bacterial strains and growth conditions

All bacterial strains used in this study are listed in table 2-1. *Bifidobacterium* strains were grown anaerobically at 37°C either in MRS broth (Biolife) supplemented with L-cysteine-hydrochloride (0.5g/l) and resazurine (2mg/l); BHI broth (Biolife) supplemented with yeast extract (5g/l), L-cysteine-hydrochloride (0.5g/l) and resazurine (2mg/l); RB broth (Hartemink *et al.*, 1996); Columbia broth (Beerens, 1990); BFM broth (Nebra and Blanch, 1999); semiselective BHI broth supplemented with mupirocin (modified method of Rada and Koc (2000)), or Iwata broth (Iwata and Morishita, 1989). All media for anaerobic growth purposes were reduced using L-cysteine-hydrochloride (0.5g/l). Preparation of media and growth experiments involving *Bifidobacterium* strains were carried out in an anaerobic chamber (COY Laboratories Products Inc., Ann Arbor, ML USA) under a gas atmosphere of 94% nitrogen and 6% hydrogen. The incubation periods were between 12 hours and three days depending on the strain. Strain purity was regularly tested by microscopy, culture morphology and PCR techniques.

Medium preparation for other bacteria included: *Corynebacterium* broth (pH 7.2-7.4) containing casein-peptone (10g/l), yeast extract (5g/l), glucose (5g/l), NaCl (5g/l), Tween80 (0.5%) and glycine (25g/l). *Lactococcus* broth GM17 (M17 broth (Terzaghi and Sandine, 1975) with lactose replaced by glucose). LB-medium (Sambrook *et al.*, 1989) was used for cultivation of *E. coli* and *B. subtilis*.

All media were prepared with distilled water and sterilised by autoclaving at 121°C for 15 minutes. For the preparation of solid medium a supplement of 14g/l agar-agar (Oxoid) was used. A stock of strains was kept at –80°C in appropriate medium containing 30% (v/v) glycerol.

Antibiotic supplements (Sigma) were used at indicated concentrations (µg/ml) to maintain resistance genes or for selection of plasmid and vector containing strains. With the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) to LB agar plates at a final concentration of 0.04mM and

40µg/ml, respectively, a blue and white colony selection of pUC18 transformants of *E. coli* XL1-Blue was possible.

Table 2-1: Bacterial strains used in this study.

Species or strain	Source or reference [a]	Growth parameters [b]	Relevant characteristics [c]
	Bifidobacteria		
B. adolescentis	DSMZ 20083^T	MRS, BHI, 37°C, aa	Na_{30}
B. angulatum	DSMZ 20098^T	MRS, BHI, 37°C, aa	Na_{30}
B. animalis	DSMZ 20104^T	MRS, BHI, 37°C, aa	Na_{30}
B. animalis	DSMZ 20105	MRS, BHI, 37°C, aa	Na_{30}
B. animalis P23	Scardovi and Trovatelli (1974)	MRS, BHI, 37°C, aa	Na_{30}
B. asteroides	DSMZ 20089^T	MRS, BHI, 37°C, aa	Na_{30}, containing pAP1
B. asteroides	DSMZ 20431	MRS, BHI, 37°C, aa	Na_{30}
B. bifidum	DSMZ 20456^T	MRS, BHI, 37°C, aa	Na_{30}
B. boum	DSMZ 20432^T	MRS, BHI, 37°C, aa	Na_{30}
B. breve	DSMZ 20213^T	MRS, BHI, 37°C, aa	Na_{30}
B. catenulatum	DSMZ 20103^T	MRS, BHI, 37°C, aa	Na_{30}
B. choerinum	DSMZ 20434^T	MRS, BHI, 37°C, aa	Na_{30}
B. coryneforme	DSMZ20216^T	MRS, BHI, 37°C, aa	Na_{30}
B. cuniculi	DSMZ 20435^T	MRS, BHI, 37°C, aa	Na_{30}
B. denticolens	DSMZ 10105^T	MRS, BHI, 37°C, aa	Na_{30}
B. dentium	DSMZ 20436^T	MRS, BHI, 37°C, aa	Na_{30}
B. gallicum	DSMZ 20093^T	MRS, BHI, 37°C, aa	Na_{30}
B. gallinarum	DSMZ 20670^T	MRS, BHI, 37°C, aa	Na_{30}
B. indicum	DSMZ 20214^T	MRS, BHI, 37°C, aa	Na_{30}
B. infantis	DSMZ 20088^T	MRS, BHI, 37°C, aa	Na_{30}
B, inopinatum	DSMZ 10107^T	MRS, BHI, 37°C, aa	Na_{30}
B. lactis	DSMZ 10140^T	MRS, BHI, 37°C, aa	Na_{30}
B. lactis UR1	LME	MRS, BHI, 37°C, aa	Na_{30}
B. longum	DSMZ 20219^T	MRS, BHI, 37°C, aa	Na_{30}
B. longum	NCC 2705	MRS, BHI, 37°C, aa	Na_{30}
B. magnum	DSMZ 20222^T	MRS, BHI, 37°C, aa	Na_{30}
B. merycicum	DSMZ 6492^T	MRS, BHI, 37°C, aa	Na_{30}
B. minimum	DSMZ 20102^T	MRS, BHI, 37°C, aa	Na_{30}
B. pseudocatenulatum	DSMZ 20438^T	MRS, BHI, 37°C, aa	Na_{30}
B. pseudolongum ssp. *globosum*	DSMZ 20092^T	MRS, BHI, 37°C, aa	Na_{30}

Table 2-1: continued.

Species or Strain	Source or reference [a]	Growth parameters [b]	Relevant characteristics [c]
B. pseudolongum ssp. *pseudolongum*	DSMZ 20099^T	MRS, BHI, 37°C, aa	Na_{30}
B. pullorum	DSMZ 20433^T	MRS, BHI, 37°C, aa	Na_{30}
B. ruminatium	DSMZ 6489^T	MRS, BHI, 37°C, aa	Na_{30}
B. saeculare	DSMZ 6531^T	MRS, BHI, 37°C, aa	Na_{30}
B. subtile	DSMZ 20096^T	MRS, BHI, 37°C, aa	Na_{30}
B. suis	DSMZ 20211^T	MRS, BHI, 37°C, aa	Na_{30}
B. thermophilum	DSMZ 20210^T	MRS, BHI, 37°C, aa	Na_{30}
CSC8 *B. longum* NCC 2705 transformant	This study	MRS, BHI, 37°C, aa	Na_{30}, Tet_{10}, containing pCSC8
***Bifidobacterium* isolates from human faecal samples**			
B. sp. CS1 to CS24	This study	MRS, BHI, 37°C, aa	Na_{30}
Other bacteria			
Bacillus subtilis 168	LME	LB, 30°C, a	Cm_{10}
Lactococcus lactis MB1363	Chr. Hansen A/S (Denmark)	GM17, 30°C, a	Tet_{25}
Corynebacterium glutamicum	ATCC13032	*Corynebacterium* broth, 30°C, a	
E. coli XL1-Blue	LME	LB, 37°C, a	Tet_{10}
E. coli JM109	Hanahan (1983)	LB, 37°C, a	
E. coli JM110	Stratagene	LB, 37°C, a	*dam, dcm*

[a] DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany
NCC: Nestec Culture Collection
ATCC: American Type Culture Collection, Manassas, USA
LME: Laboratory of Food Microbiology, ETH Zürich, Switzerland

[b] MRS, BHI, LB, GM17, *Corynebacterium* broth: see chapter 2.1 for explanation
aa: anaerobic incubation
a: aerobic incubation

[c] antibiotic resistance (µg/ml) final concentration: Na = nalidixic acid, Tet = tetracycline-hydrochloride, Cm = chloramphenicol

[T] Type strain

2.2 Plasmids

Plasmids used in this work, either for cloning purposes or transformation experiments, are listed in table 2-2.

Table 2-2: Plasmids and vectors used in this study.

Plasmid/Vector	Relevant characteristics	References
pAP1	2.141kb plasmid of *B. asteroides* DSMZ 20089^T	Kaufmann *et al.* (1997a)
pUC18	2.686kb, Ap^R, *lacZ*' cloning vector	Yanisch-Perron *et al.* (1985)
pLME201	4.827kb, Ap^R, pAP1 *Hin*dIII digested and cloned into pUC18	Kaufmann (1998)
pLME202	4.827kb, Ap^R, pAP1 *Sal*I digested and cloned into pUC18	Kaufmann (1998)
pCSC1	6.940kb, Ap^R, Tet^R, carrying the complete *tet*(W) gene from pG$^+$Host9::*tet(*W) amplified with primers cstet and cstet2.rev cloned into *Pst*I site of pLME201	This study
pCSC2	6.940kb, Ap^R, Tet^R, carrying the complete *tet*(W) gene from pG$^+$Host9::*tet*(W) amplified with primers cstet and cstet2.rev cloned into *Pst*I site of pLME201	This study
pCSC3	6.940kb, Ap^R, Tet^R, carrying the complete *tet*(W) gene from pG$^+$Host9::*tet*(W) amplified with primers cstet and cstet2.rev cloned into *Pst*I site of pLME202	This study
pCSC4	6.940kb, Ap^R, Tet^R, carrying the complete *tet*(W) gene from pG$^+$Host9::*tet*(W) amplified with primers cstet and cstet2.rev cloned into *Pst*I site of pLME202	This study
pCSC5	5.775kb, Ap^R, Cm^R, carrying the complete *cat* gene from pC194 amplified with the primers cscat2 and cscat2.rev cloned into *Xba*I/*Pst*I site of pLME201	This study
pCSC6	5.783kb, Ap^R, Cm^R, carrying the complete *cat* gene from pC194 amplified with the primers cscat2 and cscat3.rev cloned into *Pst*I site of pLME202	This study
pCSC7	5.783kb, Ap^R, Cm^R, carrying the complete *cat* gene from pC194 amplified with the primers cscat2 and cscat3.rev cloned into *Pst*I site of pLME202	This study
pCSC8	7.2kb, Ap^R, Tet^R, carrying the complete *gfpuv* gene from pGFPuv cloned into *Xba*I site of pCSC1	This study
pCSC9	7.2kb, Ap^R, Tet^R, carrying the complete *gfpuv* gene from pGFPuv cloned into *Xba*I site of pCSC1	This study
pAMT1	6.250kb, Ap^R, Cm^R, *Propionibacterium-E. coli* shuttle vector derived from *P. freudenreichii* ssp. *freudenreichii*	Stierli (2002)
pGFPuv	3.3kb, Ap^R, containing the complete *gfpuv* gene, replicating in *E. coli*	Clontech Laboratories, Palo Alto, USA (Crameri *et al.*, 1996)
pRDC102	5.658kb, Ap^R, Spec^R. *Bifidobacterium-E. coli* shuttle vector derived from *B. longum* NCC 2705	NCC (Nestec Culture Collection)
pEC-K18mob2	5.695kb, Km^R, *lacZ*', *Corynebacterium-E. coli* shuttle vector derived from *C. glutamicum* ATCC 13032	A. Tauch (unpublished)
pC194	2.901kb, Cm^R, R-plasmid from *Staphylococcus aureus*, carrying the *cat* gene, including the promoter region, replicating in *Bacillus subtilis* 168	Horinouchi and Weisblum (1982)
pG$^+$Host9::*tet(*W*)*	Tet^R, 2.1kb *Pst*I-*Pst*I fragment in pG$^+$Host9, carrying the complete *tet*(W) gene of a *Bifidobacterium* strain, including the putative promoter region, replicating in *Lactococcus lactis* MB1363	Chr. Hansen AIS, Horsholm, Denmark

Abbreviations: Ap^R, ampicillin resistance, Cm^R, chloramphenicol resistance, Tet^R, tetracycline resistance, Km^R, kanamycin resistance, Spec^R, spectinomycin resistance

2.3 General methods of DNA isolation and analysis procedures

2.3.1 Isolation of plasmid DNA

2.3.1.1 Small scale plasmid isolation from *Bifidobacterium* and *Corynebacterium*

Two modified methods were used for small scale plasmid isolation. The first method described is a modified method of Anderson and McKay (1983).

A single colony of *Bifidobacterium* or *Corynebacterium* was inoculated in 10ml of the respective liquid medium and incubated at 37°C overnight. 2ml of this culture were harvested by centrifugation (14000rpm, 4min, Eppendorf centrifuge 5417c), the cells were resuspended in 380µl of solution A (6.7% sucrose, 50mM Tris-HCl, 1mM EDTA [pH 8.0]) and prewarmed at 37°C. To lyse the cells, 96.5µl solution B (25mM Tris-HCl, 20mg/ml lysozyme [pH 8.0]) were added and incubated at 37°C for 20min. 48µl of solution C (0.25mM EDTA, 50mM Tris-HCl [pH 8.0]) and 27.5µl of solution D (20% SDS, 50mM Tris-HCl, 20mM EDTA [pH 8.0]) were added, mixed immediately and incubated at 37°C for another 10min. After mixing the solution vigorously for a few seconds, the chromosomal DNA was denatured by the addition of 27.5µl 3M NaOH and gently mixing for 10min.

With the addition of 49.5µl 2M Tris-HCl [pH 7.0] the solution was neutralised for 3min and then the DNA was precipitated for 1min after adding 71µl 5M NaCl. Proteins were extracted by adding 700µl phenol (saturated with 3% NaCl). After centrifugation (14000rpm, 4min) the upper phase was transferred into two new tubes and 650µl methylene chloride/isoamyl alcohol (24:1, v/v) were added to remove the remaining phenol. The centrifugation step was repeated, the upper phase was transferred into a new tube and 600µl isopropanol were added. After 30min of DNA precipitation on ice, the solution was centrifuged (14000rpm, 20min, 4°C). The supernatant was discarded and residual aqueous solution was dried for 10min in a vacuum centrifuge (Speed-Vac, Savant, SVC100). The resulting pellet was resuspended in 30µl autoclaved, bidistilled water. RNA was digested with 1µl RNase A (Sigma, 5mg/ml, heated for 10min at 95°C in 50mM sodium acetate [pH 5.0]).

The modified method of Frère (1994) was used as an alternative method to isolate bifidobacteria or corynebacteria plasmids at a small scale. A single colony of *Bifidobacterium* or *Corynebacterium* was inoculated in 10ml of the appropriate liquid medium and incubated at 37°C overnight. 5ml of this culture were harvested by centrifugation (3800rpm, 20min, Beckman GPR) and the pellet was resuspended in 300µl of solution 1 (50mM Tris-HCl, 10mM EDTA [pH 7.5], 100µg/ml RNase A). 0.3g autoclaved glass beads (diameter of 0.1-0.11mm, Brown) were added and the cells were broken by vortexing for 30sec at highest speed. 300µl solution 2 (1% SDS, 0.2M NaOH) were added. After incubation at room temperature for 5min, another 300µl of solution 3 (2.55M potassium-acetate [pH 4.8]) were added and the suspension was centrifuged at 14000rpm for 5min. The supernatant was distributed into two new tubes, 300µl phenol (saturated with 3% NaCl) and 300µl methylene chloride/isoamyl alcohol (24:1, v/v) were added, mixed briefly and centrifuged at 14000rpm for 5min. The upper phase was transferred into a new tube and 2 volumes of 70% EtOH (-20°C) were added to precipitate the plasmid DNA. After 1h precipitation at -20°C, the solution was centrifuged at 14000rpm for 20min at 4°C. Afterwards, the pellet was dried and resuspended with 30µl autoclaved, bidistilled water.

2.3.1.2 Large scale plasmid isolation from *Bifidobacterium*

In a modified method of Anderson and McKay (1983), using the same solutions as for small scale isolation (see chapter 2.3.1.1), the bifidobacteria cells from a 500ml MRS broth culture (1% inoculum, 37°C, anaerobically grown overnight) were harvested by centrifugation (4000xg, 15min, 4°C). The pellet was resuspended in solution A and incubated for 5min at 37°C. Cell lysis was performed by adding 7.5ml solution B and incubation at 37°C for 20min. Then 3.75ml of solution C and 2.25ml of solution D were added and left at 37°C for another 10min. After mixing briefly, chromosomal DNA was denatured by adding 2.4ml 3M NaOH and cautiously mixing for 10min. By adding 3.9ml 2M Tris-HCl [pH 7.0] (3min cautiously mixing) and 5.7ml 5M NaCl (1min cautiously mixing) the denatured DNA was precipitated. The precipitate of chromosomal DNA was removed by centrifugation (6000xg, 30min, 4°C), and the supernatant was treated with 1 volume of phenol (saturated with 3% sodium) by vigorously mixing. Phase separation was obtained by centrifugation at room temperature (Beckman GPR, 4000rpm, 20min).

The residual phenol was extracted by combining 1 volume of methylene chloride/isoamyl alcohol (24:1) with the upper phase, and the separation of the two phases was obtained by centrifugation at room temperature (4000rpm, 20min). Plasmid DNA in the aqueous upper phase was precipitated by adding 2/3 volume of isopropanol and leaving the sample at room temperature for 1h. After centrifugation at room temperature (4000rpm, 20min) the DNA pellet was washed twice with 70% ethanol (-20°C), dried and dissolved in 1ml autoclaved, bidistilled water. The solution was treated with 1µl RNase A (Sigma, 5mg/ml) for 10min at 37°C.

2.3.1.3 Plasmid isolation from *E. coli*

Small scale plasmid DNA isolation was performed following a modified procedure of Holmes and Quigley (1981).

10ml of LB broth with the appropriate antibiotics were inoculated with a single colony and grown aerobically overnight at 37°C. 1ml was harvested by centrifugation (14000rpm, 10min, Eppendorf centrifuge 5417c) and the resulting pellet was resuspended in 100µl STETL-solution (8% sucrose, 0.5% Triton X-100, 50mM Tris-HCl, 50mM EDTA [pH 8.0], 0.5mg/ml lysozyme), put on ice for 15min and boilt for 45sec and then placed back on ice for 10min. 1µl RNase A (Sigma, 5mg/ml, heated for 10min at 95°C in 50mM sodium acetate [pH 5.0]) was added. After centrifugation at 1400rpm for 10min the pellet was removed with an autoclaved tooth pick. Plasmid DNA in the supernatant was precipitated by adding 110µl isopropanol, mixed briefly, centrifuged at 14000rpm for 15min at 4°C, dried and dissolved in 25µl autoclaved, bidistilled water.

Large scale plasmid DNA isolation of *E. coli* was performed with the "Wizard® *Plus* Midipreps Purification System" (Promega, USA) according to the protocol of the manufacturer.

2.3.2 Agarose gel electrophoresis

The isolated DNA was mixed with 6x glycerol-loading buffer (Sambrook *et al.*, 1989) and separated in agarose gels using 1x TBE buffer (Sambrook *et al.*, 1989). Routine electrophoresis was performed in a 0.8% agarose gel. For linear DNA samples, a 1kb DNA ladder (Amersham Pharmacia Biotech) was used as size reference. The circular DNA standard was a supercoiled DNA ladder (Promega).
After electrophoresis (PowerPac 3000 Power Supply, Bio Rad), the agarose gels were stained in a 5µl/ml ethidium-bromide (Sigma) solution for 10min and washed for another 10min in distilled water. The DNA fragments were visualised under UV light (302nm), photographed and digitised with the "AlphaImager™" system (Alpha Innotech Corporation).

2.3.3 Methods of DNA purification

2.3.3.1 DNA isolation from low melting point agarose gels

The electrophoresis with the low melting point agarose gel was proceeded as described above at 70V. After visualisation under UV light (302nm) the DNA band of interest was cut out with a sterile knife and DNA was isolated and purified using the QIAEX II Gel Extraction Kit (Qiagen) according to the manufacturer's manual. The DNA was resuspended in autoclaved, bidistilled water.

2.3.3.2 DNA purification

Isolated or digested DNA was purified using the QIAEX II Gel Extraction Kit (Qiagen) according to the manufacturer's manual and resuspended in autoclaved, bidistilled water. PCR fragments were cleaned with the NucleoSpin® Extract 2 in 1 kit (Macherey-Nagel) according to the manual of the manufacturer.

2.3.4 Quantification of DNA

DNA was quantified by gel electrophoresis of the sample together with defined amounts of λ-DNA (Gibco) followed by densitometric analysis with the AlphaEaseFC™ software (Alpha Innotech Corporation).

2.3.5 Restriction endonuclease digests of DNA

All endonuclease digests were performed using the One Phor All Buffer *PLUS* (Amersham Pharmacia Biotech) under the conditions recommended by the supplier. Enzymes from other suppliers were used with the appropriate buffer system according to the manufacturer's recommendations. The digests were analysed by agarose gel electrophoresis.

2.3.6 Polymerase chain reactions

The PCR was performed in a total volume of 50µl containing 0.2mM each of dATP, dCTP, dGTP, dTTP (Amersham Pharmacia Biotech), 1U *Taq*-DNA-Polymerase (Amersham Pharmacia Biotech) or 1U *Pwo*-DNA-Polymerase (Roche), 5µl 10x standard PCR-buffer (Amersham Pharmacia Biotech or Roche), 1µM each of forward and reverse primers (see table 2-3) and 42µl template DNA. To prepare template DNA, 2ml of an overnight broth culture was harvested and a small portion of the cell pellet was transferred with an autoclaved tooth pick into 210µl of autoclaved, bidistilled water. Target DNA was amplified in a 0.2-ml thin-walled tube using thermocyclers (Personal Cycler, Biometra or Genius, Techne).
The standard PCR program contained an initial denaturating step at 95°C for 3min, followed by 40 cycles comprising a denaturating step at 95°C for 15sec, a 30sec annealing step at a temperature appropriate for the primer pair used and an extension step at 72°C for 2min. At the end, a final extension was performed at 72°C for 7min followed by a final hold at 4°C.

Table 2-3: Synthetic oligonucleotides used for PCR or hybridisations. Oligonucleotides were synthesised by Microsynth (Balgach, Switzerland).

Primer	Sequence (5'→3')	Position	Target gene	Annealing temperature	Reference
		Oligonucleotides used for PCR			
lm3	CGGGTGCTICCCACTTTCATG	1412-1432[1]	Genus *Bifidobacterium* 16S rDNA	60°C	Kaufmann (1998)
lm26	GATTCTGGCTCAGGATGAACG	15-35[1]	Genus *Bifidobacterium* 16S rDNA	60°C	Kaufmann (1998)
bak11w	AGTTTGATCMTGGCTCAG	8-25[1]	universal[3] 16S rDNA	60°C	Goldenberger (1997)
bak4	AGGAGGTGATCCARCCGCA	1540-1522[1]	universal[3] 16S rDNA	60°C	Greisen *et al.* (1994)
cstet	TTTGCTGCAGCTTAGTTTTTTGTAC		complete *tet(*W*)* of pG$^+$Host9 *Pst*I site	50°C	This study
cstet2.rev	TTTGGCTGCAGTACATTATCTTCTG		complete *tet(*W) of pG$^+$Host9 *Pst*I site	50°C	This study
cscat2	TTTTTCTGCAGGCACCCATTAGTTC	1093-1106[2]	complete *cat* gene of pC194	55°C	Fretz (2000)
cscat2.rev	TTTTTTCTAGAAAAGGAGAAGTCGG	2042-2029[2]	complete *cat* gene of pC194	55°C	Fretz (2000)
cscat3.rev	TTTTTCTGCAGAAAGGAGAAGTCGG	2042-2029[2]	complete *cat* gene of pC194	55°C	Fretz (2000)

[1] *E. coli* positions in the 16S rDNA according to Brosius *et al.* (1978)

[2] Position according to pC194 (EMBL accession no V01277)

[3] Universal primer for targeting most of the known bacterial 16S rDNA sequences

2.3.7 Hybridisation techniques

2.3.7.1 Labelling of DNA fragments with [γ-^{32}P]ATP

Oligonucleotides were commercially synthesised (Microsynth). 5pmoles of the respective oligonucleotide were labelled with radioactive 2500Ci/mmol [γ-^{32}P]ATP (NEN Life Science Products) using the T4-Polynucleotidekinase as described by Sambrook *et al.* (1989). The probe was purified using NAP-columns (Amersham Pharmacia Biotech) to remove non-incorporated radioactivity according to the manufacturer's instructions. Then, the probe was denatured for 10min at 95°C and kept on ice for another 10min before adding to the hybridisation solution.

2.3.7.2 Random primed labelling of DNA fragments with [α-^{32}P]dATP

In a final volume of 25µl, the linearised DNA fragment (100-500ng) was labelled with 3000Ci/mmol [α-^{32}P]dATP (NEN Life Science Products) using the random priming technique of Feinberg and Vogelstein (1983). To remove excess unincorporated radioactivity, the probe was purified using a NICK-column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Then, the probe was denatured for 10min at 95°C and kept on ice for another 10min before adding to the hybridisation solution.

2.3.7.3 Southern blotting

DNA from agarose gels was transferred to positively charged GeneScreen Plus® Charged Nylon Membranes (NEN Life Science Products) by the method of Southern (1975), using the vacuum blotting technique. The DNA was partially depurinated by soaking the agarose gels twice in 0.25M HCl under gentle rotation for 10min (Wahl *et al.*, 1979). The DNA was denatured in 0.4M NaOH for 4min before 1h of vacuum blotting also in 0.4M NaOH with a vacuum blotting equipment (Biometra). After the transfer, the membranes were washed in 2xSSC (0.3M NaCl, 0.03M sodium citrate [pH 7.0]) and dried at 80°C for 30min. Finally, the membranes were stored air-tight and light-protected at 4°C.

2.3.7.4 Colony hybridisation

Colonies were transferred onto a Colony/Plaque Screen™ Nylon Membrane (NEN Life Science Products) by direct plaque lift as described by the supplier. The membrane was placed with the colony side upwards on a 3MM paper (Whatman) soaked with lysis-solution (10mM Tris-HCl [pH 7.5], 0.25M sucrose, 30mg/ml lysozyme) and incubated for 1h at 37°C. After cell-lysis, the membrane was placed on a 3MM paper soaked with denaturation solution (0.5M NaOH, 1M NaCl). After 4min at room temperature, the membrane was transferred to a fresh 3MM paper soaked with 1M Tris-HCl [pH 8.0] for 4min. The membrane was placed for 4min on another fresh filter paper that had been soaked with 2xSSC (0.3M NaCl, 0.03M sodium citrate [pH 7.0]). The membrane was dried at 80°C for 10min and irradiated for 4min under UV light (302nm) to fix the DNA to the membrane. Finally, the membrane was stored air-tight and light-protected at 4°C.

2.3.7.5 Dot blot hybridisation

2µl (100-500ng) of isolated plasmid DNA or total DNA were mixed with 3µl of 0.2M NaOH and incubated at 37°C for 15min. After incubation, the whole DNA suspension was transferred onto a positively charged GeneScreen Plus® Charged Nylon Membrane (NEN Life Science Products), dried at 80°C for 2h and UV-crosslinked for another 5min (302nm). Finally, the membrane was stored air-tight and light-protected at 4°C.

2.3.7.6 Hybridisation of DNA probes

The membranes was incubated with 30ml prehybridisation solution (5x SSC; 5x Denhardts, 0.25mg/ml sssDNA, 0.05M sodium phosphate buffer [pH 6.5]) in a Micro-4 oven (Hybaid) at 60°C, if [γ-^{32}P]ATP labelled primer lm3 was used and at 65°C, if [α-^{32}P]dATP labelled primers were used. After 3h, the prehybridisation solution was replaced by 30ml of hybridisation solution (5x SSC, 1x Denhardts, 0.5mg/ml sssDNA, 0.04M sodium phosphate buffer [pH 6.5]) containing the labelled probe. The hybridisation was performed overnight at the same temperature as used for prehybridisation.
After the hybridisation, the membranes were washed, detected and reprobed as described by the method of Sambrook *et al.* (1989).

2.3.7.7 Membrane stripping

To remove the radioactive probe from the hybridised membranes, the membranes were incubated with 100ml 0.4M NaOH in a Micro-4 oven (Hybaid) at 45°C for 30min. After that, the NaOH-solution was discarded, replaced by 100ml washing solution (0.1% SDS, 0.1xSSC) and the membranes were incubated for 15min at room temperature. Then, the washing solution was replaced by 100ml of 0.2M Tris-HCl [pH 7.5] and incubated again for 15min at room temperature. Finally, the membranes were dried at 80°C for 10min and stored air-tight and light-protected at 4°C.

2.3.8 Ligation of DNA

The vector and the insert DNA were digested with the appropriate restriction enzymes and analysed by agarose gel electrophoresis. If necessary the digested vector DNA was additionally dephosphorylated for 1h at 37°C using calf intestine alkaline phosphatase (CIP, Amersham Pharmacia Biotech) in One Phor All Buffer *PLUS* (Amersham Pharmacia Biotech) according to the supplier's instructions. The digested vector and the insert DNA were purified by low melting point agarose gel electrophoresis. The vector and the insert DNA were mixed (molar ratio 1:5, vector/insert), incubated at 50°C for 5min, set on ice for another 3min and dried by vacuum centrifugation (Speed-Vac, Savant, SVC100). 15µl autoclaved, bidistilled water, 2µl 10mM ATP (Sigma), 2µl 10x One Phor All Buffer *PLUS* (Amersham Pharmacia Biotech) and 1µl bacteriophage T4 DNA ligase (1U/µl, Amersham Pharmacia Biotech) were added to the dried DNA mixture and incubated at 10°C overnight. The bacteriophage T4 DNA ligase was inactivated by heating to 65°C for 10min and the sample was stored at 4°C.

2.3.9 DNA sequencing

All DNA sequencing reactions were performed on the ABI PRISM 310 Genetic Analyzer (Perkin Elmer) with a ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perking Elmer) according to the supplier's instructions, with the modification of 10pmol/µl primer concentration instead of 3.2pmol/µl for cycle sequencing and only half of the recommended total volume. The primers used for cycle sequencing were commercially synthesised by Microsynth (Balgach, Switzerland) and were listed in table 2-4. Prior to sequencing, the DNA was precipitated according to the supplier’s instructions (Precipitation Microcentrifuge Tubes, Perkin Elmer) and resuspended with 15µl template suppression reagent (Perkin Elmer). The DNA sequences were further analysed as described in the following chapter.

Table 2-4: Oligonucleotide primers used for automated sequencing.

Primer	Sequence (5'→3')	Position	Target gene	Annealing temperature	Reference
cs201cat	AAAGACCCAAGCATGAACCG	1931-1950[2]	Internal forward primer for pCSC5	50°C	Fretz (2000)
cs201cat.rev	ACGAATTCGAGCTCGGTACC	2459-2440[1]	Internal reverse primer for pCSC5	50°C	Fretz (2000)
cs202cat	TGTAAAACGACGGCCAGTGC	2380-2399[1]	Internal forward primer for pCSC6/7	50°C	Fretz (2000)
cs202cat.rev	ATATGCTCGACACGTTGCC	1130-1110[2]	Internal reverse primer for pCSC6/7	50°C	Fretz (2000)

[1] *E. coli* positions according to Brosius *et al.* (1978)

[2] Position in pAP1 according to EMBL accession no Y11549

2.3.9.1 Sequence data analysis

DNA sequences were analysed with the programs provided in the GCG-package version 10.0 (Genetics Computer Group; Madison, Wisconsin, USA). DNA sequences were compared with a local GenEMBL database copy (Genbank and EMBL). Graphic representations of plasmids were drawn using the Plasmid Map Enhancer 3 program (sci.-ed software, Durham, USA) or the Omiga software (version 2.0, Rainbow Technologies, USA).

2.4 Isolation and characterisation of *Bifidobacterium* from human faeces

The human faecal samples were collected according to the partially anaerobic method of Lebet *et al.* (1998). 1g from each sample was dissolved in 90ml BHI broth and mixed until a homogenous suspension was obtained. The suspension was brought immediately into the anaerobic chamber. Serial dilutions were prepared in BHI broth, streaked on BHI agar plates and incubated anaerobically at 37°C for up to 3 days. To obtain pure cultures, single colonies of the isolates were transferred 3 times onto fresh BHI agar plates. Then, single colonies were picked (50 colonies a plate) and prepared for colony hybridisation as described in chapter 2.3.7.4. After hybridisation with the genus specific [γ-^{32}P]-labelled probe lm3 (see table 2-3), colonies that gave a positive signal were selected for further experiments. The affiliation of all bifidobacteria isolates to the genus *Bifidobacterium* was confirmed by PCR. In addition, these strains were tested for plasmid content by small scale plasmid isolation (see chapter 2.3.1.1).

2.5 Antibiotic susceptibility testing

All bifidobacteria strains from the type strain collection DSMZ and of two other strains used for transformation experiments were tested for their antibiotic susceptibility. 10ml MRS broth were inoculated with 100µl of an overnight broth culture and anaerobically incubated overnight at 37°C until an optical density (OD_{600}) of 2.0 was reached. 200µl of these cultures were added to 3ml of softagar and spread over a MRS agar plate according to the softagar overlay method. The plates were dried for about 30min. Afterwards "Etest" strips (Etest® System, AB Biodisk) were placed onto the plates (2 strips a plate) and results were determined after anaerobic incubation for 24h at 37°C. The reading of the minimal inhibitory concentrations (MIC's) of each antibiotic was performed as recommended by the manufacturer.
E. coli XL1-Blue and JM109 were also tested on LB agar plates according to the supplier's (AB Biodisk) instructions and evaluated after incubation for 24h at 37°C.

2.5.1 Softagar overlay method

The softagar overlay method was used to guarantee total anaerobiosis. For all experiments including the overlay method, 3ml of tempered (55°C) softagar (0.5% agar-agar, Oxoid) of the appropriate medium were mixed with 200µl broth culture and poured onto the appropriate agar plate. The experiments were carried out in an anaerobic chamber (COY Laboratories Products Inc., Ann Arbor, ML USA) under a gas atmosphere of 94% nitrogen and 6% hydrogen.

2.6 Transformation experiments

2.6.1 Transformation of *E. coli*

2.6.1.1 Electrotransformation of *E. coli* XL1-Blue

Competent cells of *E. coli* XL1-Blue were obtained after the method of Sambrook *et al.* (1989) using 10% glycerol solution. Best results were achieved when these cells were mixed (ratio 1:1) with *Epicurian coli*® XL1-Blue competent cells (Stratagene). In a volume of 40µl, *E. coli* XL1-Blue cells were transformed by electroporation using a Gene Pulser™ and a Pulse Controller™ apparatus (Bio-Rad Laboratories, Richmond, CA) with a field strength of 12.5 kV/cm, capacitor setting on 25µF and resistance on 200Ω following the Gene Pulser™ Electroprotocols manual, yielding a pulse duration of 4-5ms. For selecting transformants harbouring the *cat* resistance, 10µg/ml chloramphenicol was added to the LB selective medium.

2.6.1.2 Transformation of $CaCl_2$-treated *E. coli* JM109 and *E. coli* JM110 cells

E. coli JM109 competent cells were obtained from Stratagene and *E. coli* JM110 competent cells from Promega. In order to transform these $CaCl_2$-treated strains, the manufacturers' protocols were followed.

2.6.2 Electrotransformation of *Corynebacterium*

Competent cells of *Corynebacterium glutamicum* ATCC 13032 were obtained following the method of Sambrook *et al.* (1989)) for *E. coli*, using *Corynebacterium* broth (see chapter 2.1) and 15% glycerol solution. 60µl of competent corynebacteria cells were electroporated as described above for *E. coli* XL1-Blue (2.6.1.1). The selective LB medium was supplemented with 5g/l glucose and either 25mg/l kanamycin, 10mg/l chloramphenicol or 10mg/l tetracycline depending on the marker gene, which was present on the vector.

2.6.3 Electrotransformation of *Bifidobacterium*

2.6.3.1 Preparing of competent *Bifidobacterium* cells

Bifidobacteria cells were prepared for electrotransformation using different methods listed in table 2-5. The culture media were MRS or BHI broth supplemented with different carbohydrates, or modified and unmodified Iwata medium (Iwata and Morishita, 1989). For all methods, 10ml broth were inoculated with a single colony or with 100µl of an overnight broth culture and incubated anaerobically overnight at 37°C. Fresh growth medium was inoculated with different volumes of overnight cultures as indicated in table 2-5 and incubated at 37°C for 1-3h until an optical density (OD_{600}) of 0.2-0.4 was reached (early log growth phase). The cells were chilled on ice and harvested by centrifugation (Beckman, 4000xg, 15min, 4°C). The pellet was resuspended in the respective washing solution and concentrated by further washing steps along the table below. After the washing step, the pellet was resuspended in a final volume of the corresponding electroporation buffer. The cellsuspensions were brought to 10% glycerol and stored at –80°C or kept on ice until immediate use for electroporation. Viability of competent cells was determined by plating out cells that were electroporated without DNA, on a non-selective agar plate.

Table 2-5: Summary of preparation methods for competent *Bifidobacterium* strains.

Method number	Strain[a]	Growth medium[b]	Washing solution[c]	Electroporation medium[d]	Reference
1	*B. animalis* 20104^T *B. breve* 20213^T *B. catenulatum* 20103^T *B. cunniculi* 20435^T *B. gallicum* 20093^T *B. indicum* 20214^T *B. infantis* 20088^T	BHI+glycine 2.5% (w/v) [1%]	10% (v/v) glycerol+0.5M sucrose [1/10V, 1/10V]	10% (v/v) glycerol+0.5M sucrose [1%]	modified from Wells *et al.* (1993)
2	*B. angulatum* 20098^T *B. choerinum* 20434^T *B. lactis* UR1 *B. minimum* 20102^T *B. ruminantium* 6489^T	BHI [1%]	10% (v/v) glycerol+0.5M sucrose [1/10V, 1/10V]	10% (v/v) glycerol+0.5M sucrose [1%]	modified from Wells *et al.* (1993)
3	*B. animalis* 20104^T *B. animalis* P23 *B. asteroides* 20431 *B. asteroides* 20089^T	BHI [1%]	10% (v/v) glycerol [1V, 1/2V, 1/10V]	10% (v/v) glycerol [1%]	modified from Sambrook *et al.* (1989)
4	*B. animalis* 20104^T *B. animalis* 20105 *B. infantis* 20088^T	BHI+0.5M sucrose [4%]	0.5M sucrose [1/10V, 1/10V]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [0.4%]	modified from Argnani *et al.* (1996)

Table 2-5: continued.

Method number	Strain[a]	Growth medium[b]	Washing solution[c]	Electroporation medium[d]	Reference
5	*B. animalis* 20104^T *B. animalis* 20105 *B. infantis* 20088^T *B. longum* 20219^T	MRS+0.5M sucrose [4%]	0.5M sucrose [1/10V, 1/10V]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [0.4%]	Argnani *et al.* (1996)
6	*B. animalis* 20105 *B. infantis* 20088^T *B. longum* 20219^T *B. longum* NCC 2705	MRS+0.2% glucose [4%]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [1/5V, 1/5V]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [0.5%]	modified from Argnani *et al.* (1996)
7	*B. animalis* 20104^T *B. animalis* 20105 *B. longum* NCC 2705	Iwata broth [4%]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [1/5V, 1/5V]	1mM ammonium citrate buffer (pH 6.0)+0.5M sucrose [0.5%]	modified from Argnani *et al.* (1996)
8	*B. asteroides* 20431 *B. animalis* 20105 *B. longum* NCC 2705	modified Iwata broth [5%]	5mM KPP- buffer (pH 6.8) [1/2V]	MKP+0.2M raffinose buffer (pH 4.8) [0.3%]	Rossi *et al.* (2000b)

[a] Numbers indicate the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen) numbers
[b] Numbers in brackets indicate the volumes of added overnight innocultaion culture
[c] Numbers in brackets indicate the washing solution volumes of individual washing steps
[d] Numbers in brackets indicate the final volume of the competent cells with respect to the starting volume of the culture

2.6.3.2 Electroporation of *Bifidobacterium*

Bifidobacteria cells, prepared after the methods listed in table 2-5, were used for electroporation experiments. Cells and approximately 0.5-1.5µg purified and desalted plasmid DNA were mixed and placed in pre-cooled (2h on ice) electroporation cuvettes (Equibio, inter-electrode distance 0.2cm) and kept on ice for another 5min. A high-voltage (10-12.5kV/cm) electric pulse was delivered with a Gene Pulser™ and a Pulse Controller™ apparatus (Bio-Rad Laboratories, Richmond, CA) by using the 25µF capacitor and setting the pulse controller at 200Ω or 100Ω parallel resistance, yielding a pulse duration of 2.5-4.5ms. Following electroporation, bacteria were immediately diluted with 800µl of the respective culture broth (BHI or MRS with or without 0.5M sucrose or Iwata broth). Then, the bacteria were anaerobically preincubated (either in the anaerobic chamber or in anaerobic jars) as indicated in table 2-6 to allow a recovery of the cells and an expression of the antibiotic resistance.

Table 2-6: Electroporation conditions for bifidobacteria.

Method number	Pretreatment before electroporation	Volume of competent cells	Electroporation settings	Preincubation
1	5 minutes	40µl	12.5kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
2	5 minutes	40µl	12.5kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
3	stored at –80°C	40µl	12.5kV/cm, 25µF, 100Ω	Anaerobically 3h at 37°C
4	3.5h at 4°C	80µl	10kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
5	3.5h at 4°C	80µl	10kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
6	0.5h at 4°C	50µl	10kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
7	0.5h at 4°C	50µl	10kV/cm, 25µF, 200Ω	Anaerobically 3h at 37°C
8	overnight at 0°C	80µl	12.5kV/cm, 25µF, 100Ω	Anaerobically 3h at 37°C

0.1ml of the regenerated cells were directly plated on selective medium (BHI or MRS) supplemented with or without 0.5M sucrose and the appropriate antibiotics (tetracycline 10µg/ml or 20µg/ml; chloramphenicol 10µg/ml, 20µg/ml or 25µg/ml; spectinomycin 100µg/ml) depending on the resistance marker gene. The rest of the cells was harvested by centrifugation (14000rpm, 10min, Eppendorf centrifuge 5417c), resuspended in 0.1ml of the respective culture medium and plated again on the appropriate selective medium.

Alternatively, the regenerated cells were anaerobically plated using the softagar overlay method. 0.1ml of the cells were suspended in 3ml of softagar and plated out as previously described (chapter 2.5.1).

All plates were incubated anaerobically at 37°C for 3 days to 2 weeks. As negative controls competent cells were electroporated without DNA and plated onto selective medium. To ensure the viability of the prepared cells, they were plated on a non-selective medium. Recombinant bifidobacteria cells were analysed by microscopy, by plasmid typing after small scale DNA isolation, by amplification of the selection marker gene with the PCR method and hybridisation with the selection marker gene or the inserted vector DNA as a probe. In order to exclude cross-contamination by non-bifidobacteria, PCR techniques were used (see chapter 2.3).

2.7 Construction of *Bifidobacterium-E. coli* shuttle vectors

The newly constructed shuttle vectors are derivatives of pLME201 or pLME202. These plasmids were isolated in a large scale plasmid isolation from *E. coli* XL1-Blue (see chapter 2.3.1.3). 1 volume of DNA was mixed with 3 volumes of autoclaved, bidistilled water. The diluted DNA was digested with the appropriate restriction enzyme (see chapter 2.3.5) and dephosphorylated with alkaline phosphatase. The antibiotic resistance genes to be inserted were amplified by PCR (2.3.6) and digested with the same enzymes as the plasmid DNA. Both, the prepared plasmid and the amplified insert DNA were extracted after electrophoresis in low melting point agarose, purified (see chapter 2.3.3) and finally ligated to each other (chapter 2.3.8). To increase the amount of plasmid DNA, the constructed plasmids were first transformed into *E. coli* XL1-Blue cells. To verify the constructed vectors, restriction analyses were made and, furthermore, a PCR amplification of the selection marker was performed, followed by DNA sequencing and hybridisation analysis. All constructed vectors are listed in table 2-2.

2.7.1 Construction of the new vectors pCSC1, 2, 3, 4 containing the *tet(*W) gene

For construction of the shuttle vectors pCSC1 and pCSC2, the plasmid pLME201 was used as basic unit. The vectors pCSC3 and pCSC4 are derivatives of plasmid pLME202. As a first step, both plasmids pLME201 and pLME202 were linearised with the restriction enzyme *Pst*I. The amplified *tet(*W) gene, obtained with pG^{+}Host9::*tet*(W) (table 2-2) and primer pair cstet/cstet2.rev (table 2-3), was also *Pst*I digested and inserted into both parental plasmids. This resulted in four vectors, the two vectors pCSC1 and pCSC2 (descendants of pLME201) and the other two vectors pCSC3 and pCSC4 (descendants of pLME202). The new plasmid constructs differ with respect to the orientation of the *tet(*W) gene.

2.7.2 Construction of the new vectors pCSC5, 6, 7 containing the *cat* gene

The new vector plasmids were constructed in the context of a diplomawork in our laboratory (Fretz, 2000).

For constructing the shuttle vector pCSC5, the plasmid pLME201 was used. The vectors pCSC6 and pCSC7 are derivatives of plasmid pLME202. As a first step, plasmid pLME201 was linearised with the restriction enzymes *Pst*I and *Xba*I. The amplified *cat* gene, obtained with pC194 (table 2-2) and primer pair cscat2/cscat2.rev (table 2-3), was also *Pst*I and *Xba*I digested and inserted into the parental plasmid. This resulted in vector pCSC5. Plasmid pLME202 was exactly treated as described in the previous chapter with the restriction enzyme *Pst*I, and ligated with the *Pst*I digested PCR product (primer pair cscat2/cscat3.rev), containing the *cat* gene. This yielded the new vectors pCSC6 and pCSC7, which differ with respect to the orientation of the *cat* gene.

2.7.3 Construction of the new vectors pCSC8 and pCSC9 containing the *gfpuv* gene

The basis of these new vectors was plasmid pCSC1, which had been linearised with the restriction enzyme *Xba*I. The *gfpuv* gene had been excised from plasmid pGFPuv (see table 2-2) with the restriction endonucleases *Xba*I and *Spe*I. The *gfpuv* gene was inserted into the linearised pCSC1 vector producing the new shuttle vectors pCSC8 and pCSC9, which differ with respect to the orientation of the *cat* gene.

3 Results

The endeavour of this thesis was the development of a new transformation system for bifidobacteria. To set up the experiments, a new appropriate plasmid preferably of human origin had to be found for vector construction. Then convenient antibiotic selection marker genes had to be chosen and suitable host strains with respective antibiotic susceptibilities had to be determined.
During the experiments, contaminated *Bifidobacterium* strain cultures were a serious problem. Therefore, the chapter 3.1 describes experiments for easy detection of contaminated cultures and attempts to avoid them in using specific selective media.

3.1 Strain maintenance and control

All *Bifidobacterium* species are considered to be obligately anaerobic (Biavati *et al.*, 1992) and the exclusion of oxygen is necessary to study them. There exist some oxygen tolerant *Bifidobacterium* strains which grow in liquid medium at low oxygen concentrations (De Vries and Stouthamer, 1967) and Meile *et al.* (1997) described in his study the *B. lactis* strain which even grows in liquid medium at an atmospheric oxygen concentration of 10%. Nevertheless, studying bifidobacteria requires working in an anaerobic chamber. Some of the negative aspects of using an anaerobic chamber is the difficulty to maintain sterile conditions and the risk of contamination. In order to minimise such contaminations and to maintain pure cultures of bifidobacteria different selective media were tested as described in chapter 3.1.2. Furthermore, all cultures were routinely checked by microscopy on homogeneity and by specific PCR with *Bifidobacterium* genus specific primer pairs. This PCR strategy is shown in the following chapter.

3.1.1 Selective PCR to monitor purity of *Bifidobacterium*l cultures

Working with bifidobacteria comprises the routine control of cultures. Usually the well-established phosphoketolase assay to identify bifidobacteria is used for this purpose, but the recently published study of Meile *et al.* (2001) showed that the fructose 6-phosphat-phosphoketolase enzyme does not exclusively exists in bifidobacteria. Hence, the phosphoketolase assay is no guarantee for the absence of contamination. Another possibility to check cultures is to monitor the homogeneity of the culture with microscopy. Another, more sensitive method is PCR. Kaufmann (1998) developed a *Bifidobacterium* genus specific nucleotide probe named lm3 on the basis of the 16S rRNA target gene region. He designed the new primer pair lm3/lm26 (see table 2-3) with the purpose to use it for the identification of bifidobacteria by PCR techniques. His experiments revealed that a 1.35-kb fragment from the 16S rRNA was amplified with all *Bifidobacterium* species whereas no fragment could be amplified with all other tested bacteria. The developments of the present study are based on these results reported by Kaufmann (1998).

A new PCR strategy was developed using two PCR systems with the same culture as a target. First, the culture was tested for the presence of *Bifidobacterium* with the specific primer pair lm3/lm26. Subsequently, the purity of the culture was checked with specific PCR primers which produce a distinct fragment with possible contaminating bacteria. With bifidobacteria in contrast, no fragment should be amplified. Hence, an amplification of a fragment with these primers explicitly refers to a contamination. For this reason, the primer pair bak4/bak11w (see table 2-3) was selected which produces a 1.4-kb PCR product with bacterial 16S rRNA. An example for such a PCR is shown in figure 3-2.

The 16S rRNA target region of the primers bak11w and lm26 is almost the same. While lm26 is supposed to anneal with bifidobacteria, bak11w should not use bifidobacterial 16Sr RNA as target. As a PCR primer containing 1 or more sequence mismatches is not able to anneal to the target sequence under stringent conditions, no PCR product can be produced. Therefore, a bifidobacterial 16S rDNA gene sequence comparison with the probe bak11w was made to search sequence mismatches and to confirm the assumption that no PCR fragment can be amplified with bak11w/bak4 from bifidobacteria. In figure 3-1 the alignment the probe bak11w and of all bifidobacterial 16S rDNA sequences of the GenEMBL database is shown.

	[a]8' 25' 33'	Possible / observed[b] mismatches	
bak11w	AGTTTGATCMTGGCTCAG		
lm26	GATTCTGGCTCAG GATGAACG		
B. adolescentis ATCC 15703 {BARR16SA1}	GGTTCGATTCTGGCTCAG GATNAACG	3	3
B. angulatum ATCC 27535 {D86182}	TTCGATTCTGGCTCAG GATGAACG	3	2
B. animalis ATCC 25527 {BA16SRRNA}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. asteroides ATCC 29510 {BARR16SB1}	GGTTTGATTCTGGCTCAG GATGAACG	2	2
B. bifidum DSMZ 20456 {S83624}	AGTTTGATNNTGGCTCAG GATGAACG	1	0
B. boum JMC 1211 {D86190}	CGATTCTGGCTCAG GATGAACG	3	2
B. breve ATCC 15698 {Bb16srrn}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. breve ATCC 15700^T {Bbrr16sc1}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. catenulatum ATCC 27539 {BCRR16SD1}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. choerinum ATCC 27686 {D86186}	CGATTCTGGCTCAG GATGAACN	3	2
B. coryneforme ATCC 25911 {BCRR16SE}	GGTTTGATTCTGGCTCAG GATGAACG	2	2
B. cunniculi ATCC 27916 {BCRR16SF}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. denticolens DSMZ 10105 {D89331}	TTTCGATTCTGGCTCAG GATGAACG	4	3
B. dentium ATCC 27534^T {D86183}	TTTGATCATGGCTCAG GATGAACG	1	0
B. dentium ATCC 15423 {BDRR16SG}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. gallicum JMC 8224 {D86189}	CGATTCTGGCTCAG GATGAACG	4	2
B. gallinarum JMC 6291 {D86191}	CGATTCTGGCTCAG GATGAACG	4	2
B. globosum ATCC 25865 {D86194}	TTTGATCATGGCTCAG GATGAACG	1	0
B. globosum ATCC 25865 {BGRR16SH}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. indicum ATCC 25912 {BIRR16SI}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. indicum ATCC 25912 {D86188}	TTTGATCATGGCTCAG GATGAACG	1	0
B. infantis ATCC 15697^T {D86184}	TTTGATCATGGCTCAG GATGAACG	1	0
B. infantis ATCC 15967^T {Bi16srrna}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. infantis ATCC 15677 {BIRR16SJ}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. inopinatum DSMZ 10107 {Ab029087}	AGTTTGATCATGGCTCAG GACGAACG	0	0
B. inopinatum DSMZ 10107 {D89332}	TTTCGATTCTGGCTCAG GATGAACG	4	3
B. lactis DSMZ 10140 {Bl16srrn1}	GATTCTGGCTCAG GATGAACG	4	1
B. longum ATCC 15708 {BL10152}	GGTTCGATTCTGGCTCAG GATGAANG	3	3
B. longum ATCC 15705^T {BLRR16SK}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. magnum ATCC 27540 {D86193}	TTTGATCATGGCTCAG GATGAACG	1	0
B. magnum ATCC 27540 {BMRR16SL}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. minimum ATCC 27538 {BMRR16SM}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. pseudocatenulatum JMC 1200 {D86187}	GTTTCGATTCTGGCTCAG GATGAACG	4	4
B. pseudolongum ATCC 25 {BPRR16SN}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. pullorum JMC 1214 {D86196}	AGTTTGATCATGGCTCAG GATGAACG	0	0
B. saeculare DSMZ 6531^T {D89328}	GTTTCGATTCTGGCTCAG GATGAACG	4	4
B. saeculare DSMZ 6533 {D89330}	TTCGATTCTGGCTCAG GATGAACG	4	2
B. subtile DSMZ 20096^T {D89378}	TTTCGATTCTGGCTCAG GATGAACG	4	3
B. subtile JMC 7109 {D89329}	GTTTCGATTCTGGCTCAG GATGAACG	4	4
B. suis ATCC 27533 {BSRR16SO}	GGTTCGATTCTGGCTCAG GATGAACG	3	3
B. thermophilum ATCC 25525 {BT10151}	GGTTCGATTCTGGCTCAG NATGAACG	3	3
B. thermacidophilum {Ab016246}	AGTTTGATCATGGCTCAG GATGAACG	0	0

[a]: *E. coli* positions in the 16S rDNA according to Brosius *et al.* (1978)

[b]: due to incomplete sequence data

{} GenEMBL accession numbers

DSMZ Deutsche Sammlung für Mikroorganismen und Zellkulturen; JCM Japan collection of microorganism; ATCC American type culture collection

Figure 3-1: Alignment of probe bak11w with bifidobacterial 16S rDNA sequences. Numbers in parenthesis indicate GenEMBL accession numbers. Blue marked bases mark mismatches to the probe bak11w. The yellow marked sequences are of the same subspecies.

Of all 42 aligned sequences the sequences of 9 strains do not show any mismatches to the probe bak11w. Amazingly, the GenEMBL database contains for 5 of these strains (*B. globosum* ATCC 25865, *B. indicum* ATCC 25912, *B. infantis* ATCC 15697, *B. inopinatum* DSMZ 10107, *B. magnum* ATCC 27540) a second sequence containing 3 mismatches to probe bak11w.

The *B. bifidum* strain sequence contains several unidentified nucleotides (N) at the relevant region. Hence, no statement about the annealing possibility of bak11w can be made.

The remaining sequences of *B. pullorum, B. dentium* and *B. thermacidophilum* showed really no mismatches to the probe bak11w.

All bifidobacteria listed in table 2-1 were tested several times for their purity by this new PCR strategy. Only the *B. inopinatum* strain gave positive PCR signals for both primer pairs in every PCR approach made (see figure 3-2, lane 27 and 28). The *B. inopinatum* culture was checked furthermore by microscopy but no contaminant bacteria could be detected. All the other strains gave at least in one PCR approach negative signals for the primer pair bak4/bak11w. Therefore, the new PCR strategy was regarded as useful for the routine control of purity of bifidobacteria cultures with the exception of *B. inopinatum*.

3.1.2 Testing of various selective media for *Bifidobacterium*

In order to obtain and maintain pure *Bifidobacterium* cultures, the selective media Columbia broth, RB broth, BFM broth and BHI + mupirocin broth (see chapter 2.1) were tested (Fretz, 2000).

Most contaminations in anaerobic chambers are caused by enterococci and clostridia. Therefore, a selective medium was required to discriminate bifidobacteria from these contaminants. The selectivity of the media tested was determined by selective PCR (see chapter 3.1.1) in addition to microscopy. First, PCR fragments had been amplified with *Bifidobacterium* cultures grown on MRS agar. In figure 3-2 and figure 3-3 the specific PCR products of some tested *Bifidobacterium* type strains, grown on MRS agar plates, are shown. If a fragment with the primer pair lm3/lm26 could be produced, the tested culture contained bifidobacteria. If a fragment with the primer pair bak11w/bak4 was visible, the tested culture contained contaminant bacteria.

The contaminated cultures were cultivated on selective media and afterwards another PCR approach was made to see whether the selective medium promoted the growth of bifidobacteria in the culture. In figure 3-4 and figure 3-5 the selective PCR of several *Bifidobacterium* type strains cultured in various selective media are shown.
In all figures the odd numbers indicate PCR with primer pair lm3/lm26 and all even numbers show PCR with primer pair bak11w/bak4.

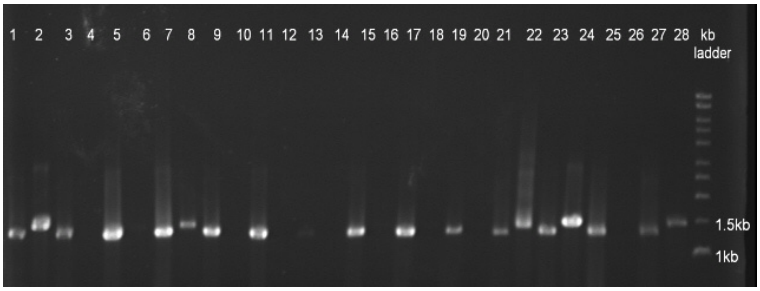


Figure 3-2: Specific PCR with bifidobacterial type strains of DSMZ (see table 2-1). The odd numbers indicate PCR products with primer pair lm3/lm26 and all even numbers show PCR products with primer pair bak11w/bak4. Lane 1-2: *B. adolescentis*. Lane 3-4: *B. animalis*. Lane 5-6*: B. asteroides* DSMZ 20089^T. Lane 7-8: *B. asteroides* DSMZ 20431. Lane 9-10: *B. breve*. Lane 11-12: *B. catenulatum*. Lane 13-14: *B. choerinum*. Lane 15-16: *B. cuniculi*. Lane 17-18: *B. dentium*. Lane 19-20: *B. gallicum*. Lane 21-22: *B. gallinarium*. Lane 23-24*: B. indicum*. Lane 25-26: *B. infantis*. Lane 27-28: *B. inopinatum*. Kb-ladder (Pharmacia).

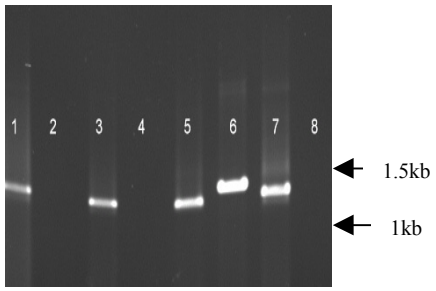


Figure 3-3: Specific PCR with bifidobacterial type strains of DSMZ (see table 2-1). The odd numbers indicate PCR with primer pair lm3/lm26 and all even numbers show PCR with primer pair bak11w/bak4. Lane 1-2: *B. lactis*. Lane 3-4: *B. longum*. Lane 5-6*: B. magnum*. Lane 7-8: *B. merycicum*.

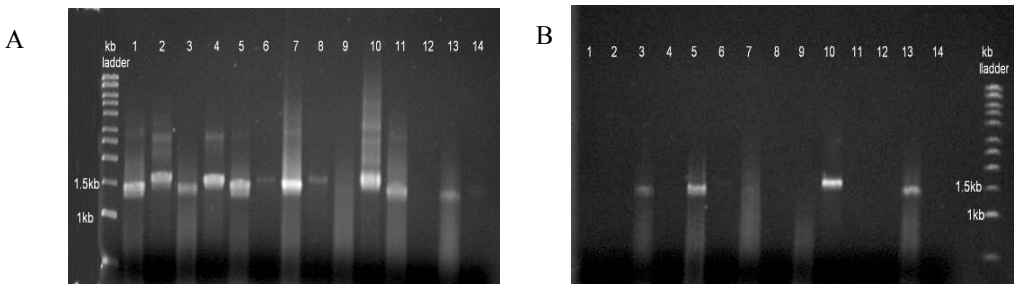


Figure 3-4: Specific PCR with bifidobacterial type strains of DSMZ (see table 2-1) cultured on Columbia medium or BFM medium. The odd numbers indicate PCR with primer pair lm3/lm26 and all even numbers show PCR with primer pair bak11w/bak4.

A: Columbia medium: Lane 1-2: *B. adolescentis*. Lane 3-4: *B. magnum*. Lane 5-6*: B. asteroides* DSMZ 20431. Lane 7-8: *B. dentium*. Lane 9-10: *B. gallinarium*. Lane 11-12: *B. asteroides* DSMZ 20089^T. Lane 13-14: *B. pseudocatenulatum*. Kb-ladder (Pharmacia).

B: BFM medium: Lane 1-2: *B. adolescentis*. Lane 3-4: *B. asteroides* DSMZ 20089^T. Lane 5-6*: B. asteroides* DSMZ 20431. Lane 7-8: *B. dentium*. Lane 9-10: *B. gallinarium*. Lane 11-12: *B. magnum*. Lane 13-14: *B. pseudocatenulatum*. Kb-ladder (Pharmacia).

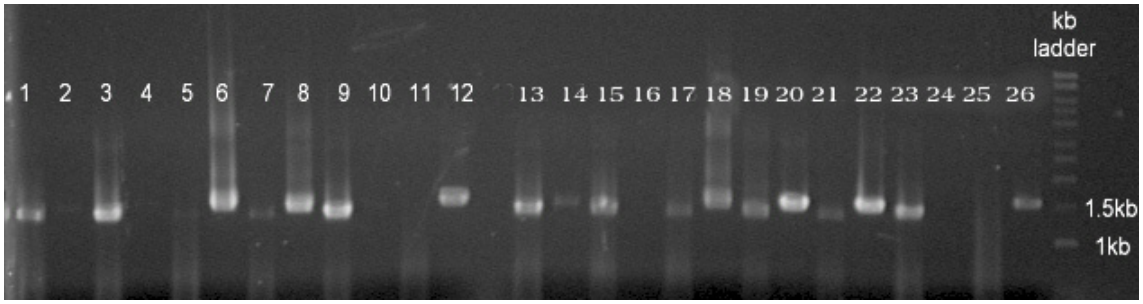


Figure 3-5: Specific PCR with bifidobacterial type strains of DSMZ (see table 2-1) cultured on RB medium (Lane 1-12) or BHI + mupirocin medium (Lane 13-26) respectively. The odd numbers indicate PCR with primer pair lm3/lm26 and all even numbers show PCR with primer pair bak11w/bak4. Lane 1-2: *B. asteroides* DSMZ 20431. Lane 3-4: *B. dentium*. Lane 5-6*: B. gallinarium*. Lane 7-8: *B. inopinatum*. Lane 9-10: *B. lactis*. Lane 11-12: *B. magnum*. Lane 13-14: *B. asteroides* DSMZ 20431. Lane 15-16: *B. dentium*. Lane 17-18: *B. gallinarium*. Lane 19-20: *B. indicum*. Lane 21-22: *B. inopinatum*. Lane 23-24*: B. lactis*. Lane 25-26: *B. magnum*. Kb-ladder (Pharmacia).

In figure 3-2 (lane 1/2, lane 7/8, lane 21/22, lane 23/24 and lane 27/28) and 3-3 (lane 5/6), PCR fragments amplified with primer pair bak11w/bak4 are visible for 6 DSMZ type strains (*B. adolescentis, B, asteroides* DSMZ 20431*, B. gallinarium, B. indicum, B. inopinatum and B. magnum*). The corresponding cultures were likely to contain contaminant bacteria. Another PCR approach was carried out with the same cultures grown in selective media.

As shown in figure 3-4 (A) and 3-5, the cultures which were regarded as contaminated in the first PCR also showed PCR fragments with primer pair bak11w/bak4 after being grown on the selective Columbia medium, RB medium and BHI + mupirocin medium. The tested pure strains *B. asteroides* DSMZ 20089^T, *B. dentium* and *B. pseudocatenulatum* produced no PCR amplicon with primer pair bak11w/bak4 (figure 3-4 (A) lane 11/12, lane 13/14, figure 3-5 lane 3/4, lane 15/16). The BFM medium was less suitable. Strains like *B. adolescentis, B. dentium* and *B. magnum* could not grow on this medium and therefore no PCR fragment is visible (see figure 3-4 (B) lanes 1/2, 7/8 and 11/12). Because the selective media tested showed no real improvement in separating bifidobacteria from contaminant bacteria and are difficult and time consuming to prepare, BHI or MRS media were used in the following experiments.
Contaminated cultures were discarded.

3.2 *Bifidobacterium* from human faecal samples

The basis of a transformation system is the creation of a suitable, high copy number plasmid vector which is able to replicate in bifidobacteria. The easiest way to construct a vector is to begin the construction with a plasmid derived from bifidobacteria which ensures the existence of a suitable origin of replication. Bifidobacteria plasmids of human origin were preferred since most of the probiotic research is done with human *Bifidobacterium* strains. Therefore, this work started with the isolation of bifidobacteria from human faecal samples and their screening for extrachromosomal DNA.

3.2.1 Isolation of *Bifidobacterium* from human faecal samples

Faecal samples from two different human volunteers were diluted and plated on BHI agar plates to isolate single colonies. Bifidobacteria were screened by the method of colony hybridisation with the genus specific [γ-^{32}P]-labelled DNA probe lm3 (Kaufmann *et al.*, 1997b). Afterwards the isolates which showed a positive hybridisation signal were confirmed as pure cultures of bifidobacteria by a selective PCR. An example for the results of colony hybridisation is shown in figure 3-6. All the results are summarised in table 3-1.

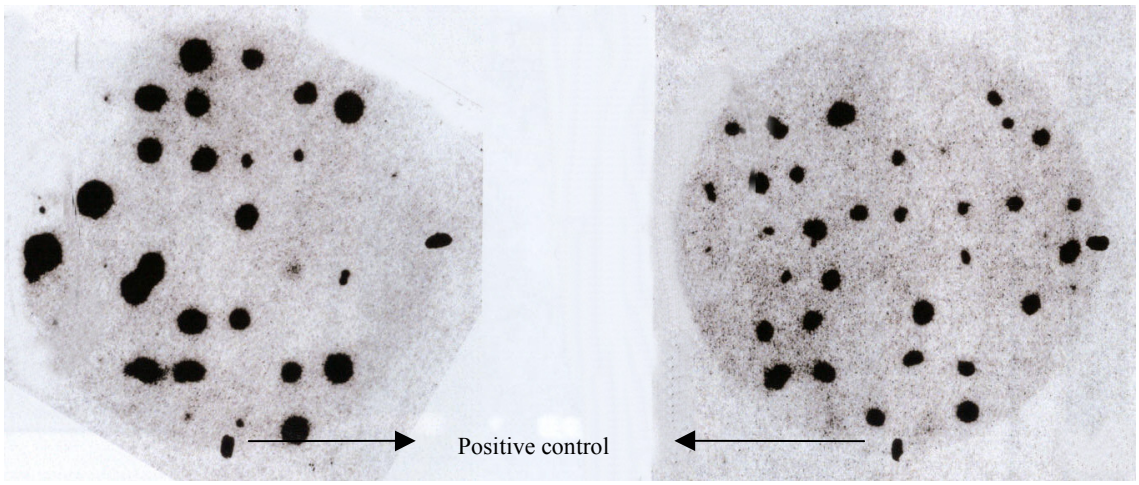


Figure 3-6: Colony hybridisation of human faecal isolates from BHI agar plates using *Bifidobacterium* genus specific [γ-^{32}P]-labelled probe lm3. Arrows indicate *B. lactis* cells as positive control.

Table 3-1: Summarised results from isolation on BHI agar plates, hybridisation and PCR confirmation of bifidobacteria in human faecal samples.

Isolated strains from human faecal samples	**Positive colonies after hybridisation**	**Positive colonies after PCR**
700	160	24
100%	23%	3.5%

700 bacteria cultures have been isolated from human faecal samples. 160 strains gave positive signals after colony hybridisation with the bifidobacteria genus specific [γ-^{32}P]-labelled probe lm3. Finally, only 24 strains could be confirmed as pure bifidobacteria cultures with the selective PCR method using the two primer pairs lm3/lm24 and bak4/bak11w as described in chapter 3.1.1. These 24 strains have been named CS1 to CS24.

3.2.2 Screening for plasmids in *Bifidobacterium* isolates from human faecal samples

The 24 isolated strains of bifidobacteria were examined for plasmid content. 14 out of the 24 strains contained plasmids with an estimated size of 8kb to >10kb. Some plasmid isolations showed similar plasmid profiles (lanes 8 and 9, lanes 10 to 13). The various bands of a plasmid isolation could represent only one plasmid with its open circular and covalently closed circular forms or could represent different plasmids. The results of this screening are summarised in table 3-2 and the small scale plasmid isolations from all strains tested are shown in figure 3-7.

Transformation vectors should be as small as possible because the segregational stability of the vector and transformation efficiency is negatively correlated with the size of the plasmid (Rossi *et al*., 1998). Bigger plasmids used for vector constructions should be reduced to the essential replication functions this could require time consuming deletion experiments. Most plasmids of this experiment have an estimated size of over 8kb (see lanes 2-7, 10-13 and 18) and were therefore regarded as too large to be convenient for further vector constructions.

Table3-2: Summary of the plasmid screening in bifidobacteria isolated from human faeces.

Strain	Containing plasmids	Approximate plasmid size	Chosen as vector
CS1; CS14-17; CS19-24	no	none	no
CS2-7; CS10-13; CS18	yes	8kb	no
CS2-13	yes	>10kb	no

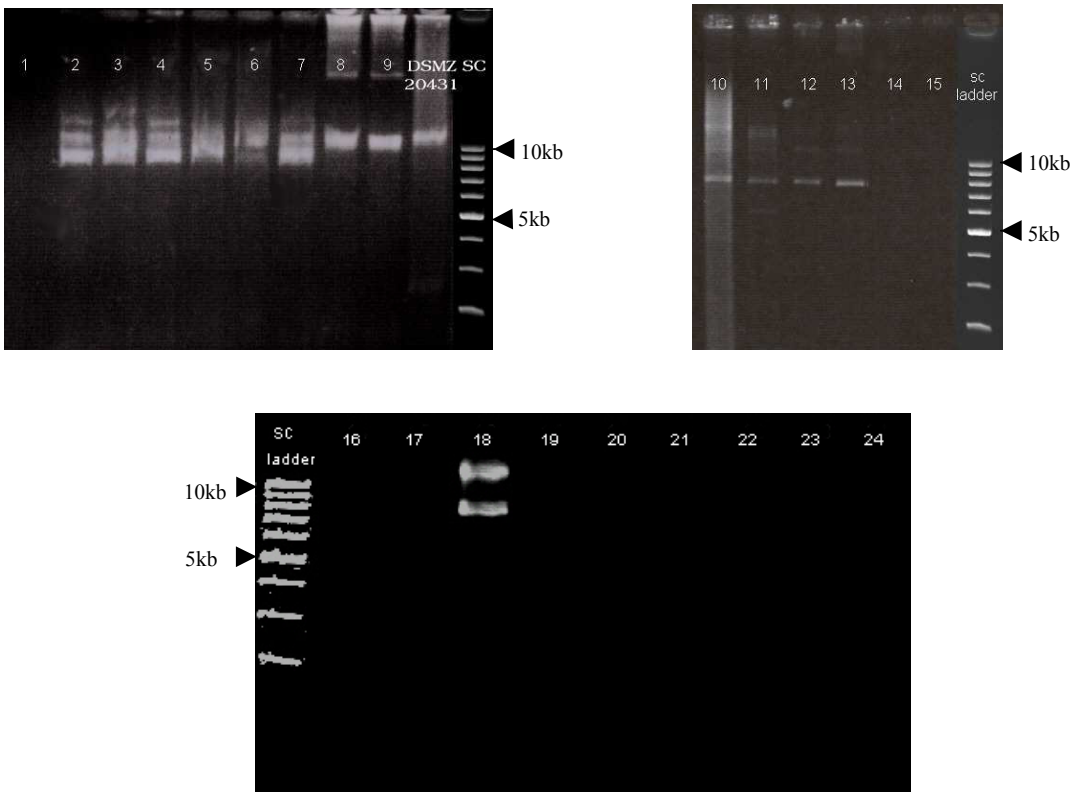


Figure 3-7: Plasmidscreening of bifidobacteria, isolated from human faecal samples. Lane 1-24: Plasmid DNA isolated from bifidobacterial isolates CS1 to CS24. Sc-ladder: supercoiled DNA ladder (2-10kb in 1-kb steps, Promega).

3.3 Antibiotic susceptibilities of *Bifidobacterium*

To construct a cloning vector, an appropriate selection marker is essential. The most common selection markers are antibiotic resistance genes. In this work the decision fell on two potential marker genes: the chloramphenicol-acetyltransferase gene (*cat*) from plasmid pC194 which was already successfully used by other research groups (Matteuzzi *et al.*, 1990; Argnani *et al.*, 1996; Rossi *et al.*, 1996; Rossi *et al.*, 1997; Rossi *et al.*, 1998), and the *tet*(W) gene (chapter 1.5.3.1). To identify suitable strains for transformation experiments all bifidobacteria strains listed in table 2-1 were screened for their minimal inhibitory concentration for chloramphenicol and tetracycline. The culture medium for the "Etests" was no standard medium as recommended by the supplier, but the same MRS medium as used for the electroporation experiments. The MIC's of chloramphenicol and tetracycline are shown in table 3-3.

Table 3-3: Antibiotic susceptibility of *Bifidobacterium* type strains, *B. longum* NCC 2705 and *B. animalis* DSMZ 20105 to chloramphenicol and tetracycline on MRS agar.

Strain	DSMZ number	MIC's of chloramphenicol µg/ml	MIC's of tetracycline µg/ml
B. adolescentis	DSMZ 20083^T	**0.38**	**0.25**
B. angulatum	DSMZ 20098^T	**0.75**	**0.25**
B. asteroides	DSMZ 20089^T	**0.38**	**0.64**
B. asteroides	DSMZ 20431	**1**	**0.16**
B. bifidum	DSMZ 20456^T	**0.50**	**1.5**
B. breve	DSMZ 20213^T	**0.75**	**0.125**
B. catenulatum	DSMZ 20103^T	**0.5**	**0.38**
B. choerinum	DSMZ 20434^T	**1**	**0.38**
B. coryneforme	DSMZ20216^T	**2**	**0.25**
B. cuniculi	DSMZ 20435^T	**1**	**2**
B. denticolens	DSMZ 10105^T	**2**	**0.25**
B. dentium	DSMZ 20436^T	**1**	**0.50**
B. indicum	DSMZ 20214^T	**0.28**	**0.75**
B. infantis	DSMZ 20088^T	**0.5**	**1.5**
B. lactis	DSMZ 10140^T	**0.047**	**0.064**
B. longum	DSMZ 20219^T	**2**	**0.5**
B. magnum	DSMZ 20222^T	**3**	**0.75**
B. merycicium	DSMZ 6492^T	**3**	**0.25**
B. minimum	DSMZ 20102^T	**1.5**	**0.5**
B. pseudocatenulatum	DSMZ 20438^T	**0.38**	**16**

Table 3-3: continued.

Strain	DSMZ number	MIC's of chloramphenicol μg/ml	MIC's of tetracycline μg/ml
B. pseudolongum ssp. *globosum*	DSMZ 20092^T	**1**	**12**
B. pseudolongum ssp. *pseudolongum*	DSMZ 20099^T	**ng**	**0.5**
B. pullorum	DSMZ 20433^T	**8**	**0.38**
B. ruminatium	DSMZ 6489^T	**0.5**	**0.25**
B. saeculare	DSMZ 6531^T	**0.75**	**0.125**
B. subtile	DSMZ 20096^T	**2**	**0.75**
B. suis	DSMZ 20211^T	**0.5**	**64**
B. thermophilum	DSMZ 20210^T	**1.5**	**128**
B. longum	NCC 2705	**0.38**	**0.125**
B. animalis	DSMZ 20105	**1.5**	**2**

ng: no growth

From the 29 strains tested, 24 strains showed MIC's for both antibiotics of less than 5μg/ml, and 1 strain of less than 10μg/ml. Due to this high antibiotic susceptibility, these 25 strains were interesting for transformation experiments. The other 4 strains showed MIC's of more than 10μg/ml for one or both antibiotics and were therefore not used for further transformation experiments. Noticeably, *B. lactis* showed about 10 fold lower inhibitory concentrations as all of the other tested *Bifidobacterium* strains. For *B. longum* NCC 2705 and *B. animalis* DSMZ 20105, which were used as host strains in previous reports of transformation experiments (Argnani *et al*., 1996; Rossi *et al*., 1997), low MIC's of chloramphenicol and tetracycline were measured.

3.4 *Bifidobacterium-E. coli* shuttle vector constructions

The ideal shuttle vector is a small plasmid with the ability to replicate in bifidobacteria as well as in *E. coli*. As shown in chapter 3.2.2, no human faecal *Bifidobacterium* strain contained plasmids small enough to be suitable for vector constructions without deletion experiments. Therefore, the *Bifidobacterium-E. coli* chimeric plasmids pLME201 and pLME202 (see table 2-2) were used as origins for the new vector constructions. The plasmids pLME201 and pLME202 were constructed in our laboratory with the *B. asteroides* plasmid pAP1 and the *E. coli* plasmid pUC18 (Kaufmann, 1998).

The newly constructed plasmids of this study were confirmed by restriction analysis and Southern hybridisations with the inserted, [α-^{32}P]-labelled resistance gene as a probe (data not shown). Figures 3-8 and 3-9 show a scheme of the vector construction strategy. The inserted antibiotic resistance gene, which had been amplified by PCR, was confirmed by complete sequencing of the resistance gene.

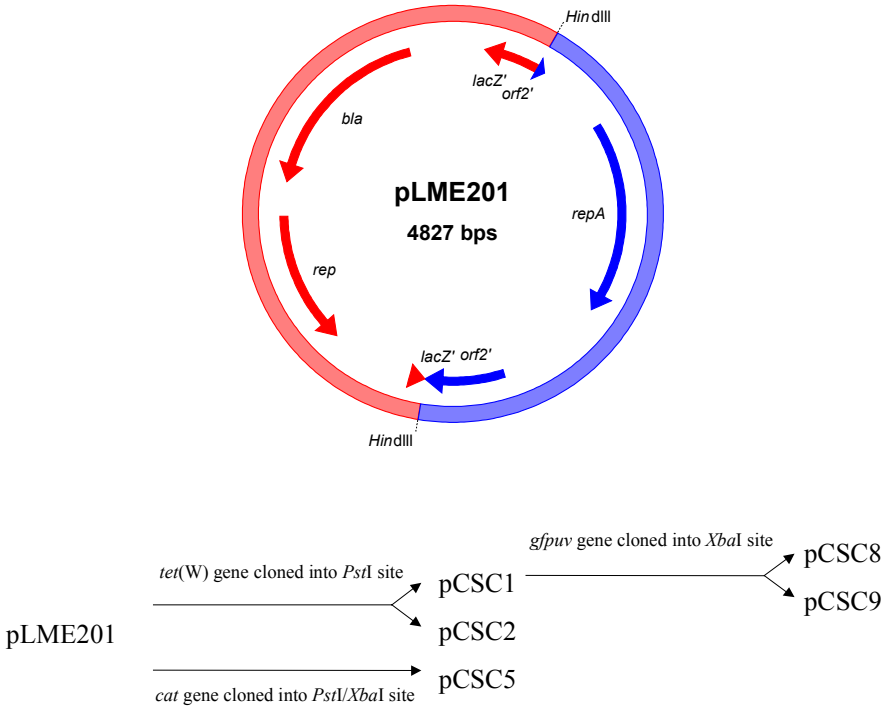


Figure 3-8: Physical map of plasmid pLME201. *RepA*: putative replicase gene of pAP1; *orf2'*: truncated *orf2* of pAP1, emerged because of the insertion of pUC18; *rep*: replication region of pUC18; *lacZ'*: truncated gene coding for β-galactosidase, including the multiple cloning site of pUC18 and *bla*: gene coding for β-lactamase confers resistance to ampicillin. Scheme of the vector construction strategy for vectors pCSC1, 2 and 5 derivatives of the parental plasmid pLME201. Resistance genes were cloned into single restriction sites (*Pst*I, *Xba*I).

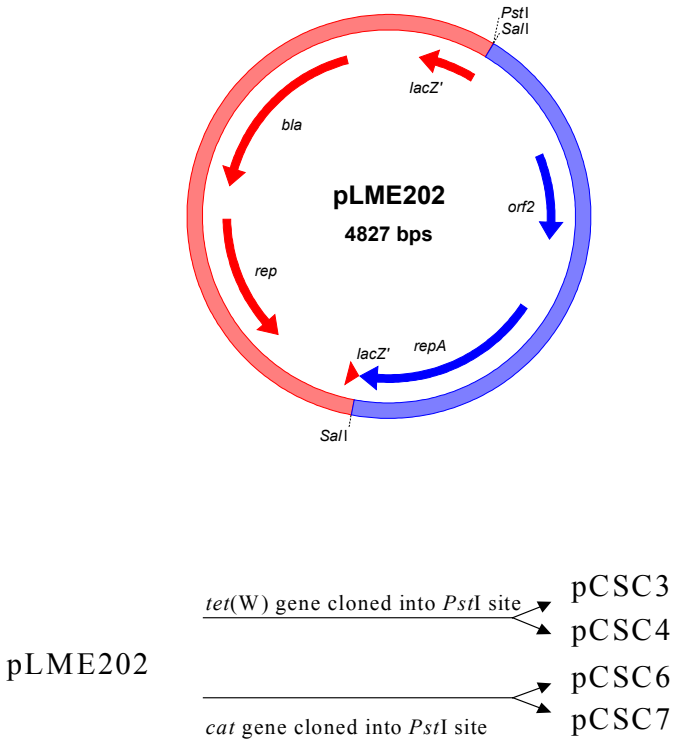


Figure 3-9: Physical map of plasmid pLME202. *RepA*: putative replicase gene of pAP1; *orf2*: cryptic gene of pAP1; *rep*: replication region of pUC18; *lac*Z': truncated gene coding for β-galactosidase, including the multiple cloning site of pUC18 and *bla*: gene coding for β-lactamase confers resistance to ampicillin. Scheme of the vector construction strategy for vectors pCSC3, 4, 6 and 7 derivatives of the parental plasmid pLME202. Resistance genes were cloned into single restriction sites (*Pst*I).

3.4.1 Vectors pCSC1, pCSC2 and pCSC5: derivatives of plasmid pLME201

Plasmid pLME201 contains the *Hin*dIII linearised pAP1 plasmid from *B. asteroides* DSMZ 20089^T, cloned into pUC18 at the *Hin*dIII site (Kaufmann, 1998). The *tet*(W) gene was amplified from pG$^+$Host9::*tet*(W) (see table 2-2) with primer pair cstet/cstet2.rev (table 2-3) and excised with *Pst*I. Then it was ligated into the *Pst*I site, located in the pUC18 part of pLME201. This resulted in plasmids pCSC1 and pCSC2 which only differ in the respective orientation of the *tet*(W) gene. Plasmid pCSC5 consists of the amplified *cat* gene from pC194 using primer pair cscat2/cscat2.rev, and pLME201 which were assembled at the *Pst*I/*Xba*I sites of pUC18. The physical maps of the obtained vectors are shown in figure 3-10 and 3-11.

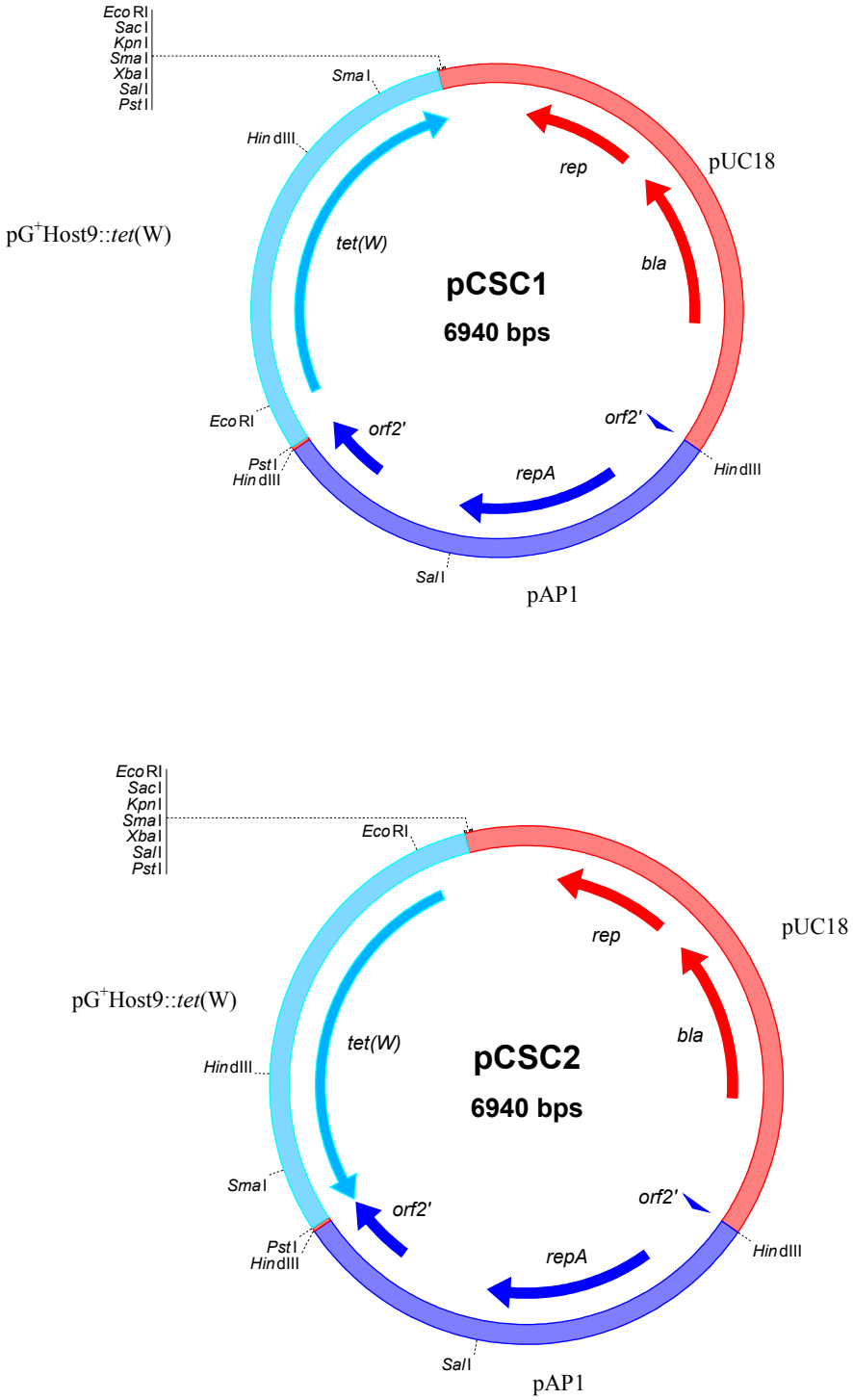


Figure 3-10: Physical maps of the plasmids pCSC1 and pCSC2. Assemblies of plasmid pLME201 and the amplified *tet*(W) gene from plasmid pGHost9::*tet*(W) at the *Pst*I restriction site. *rep*A: putative replicase gene of pAP1; *orf2'*: truncated *orf2* of pAP1, emerged because of the insertion of pUC18; *rep*: replication region of pUC18; *bla*: gene coding for β-lactamase confers resistance to ampicillin; *tet*(W): ribosome-protection tetracycline resistance determinant from a *Bifidobacterium* sp.

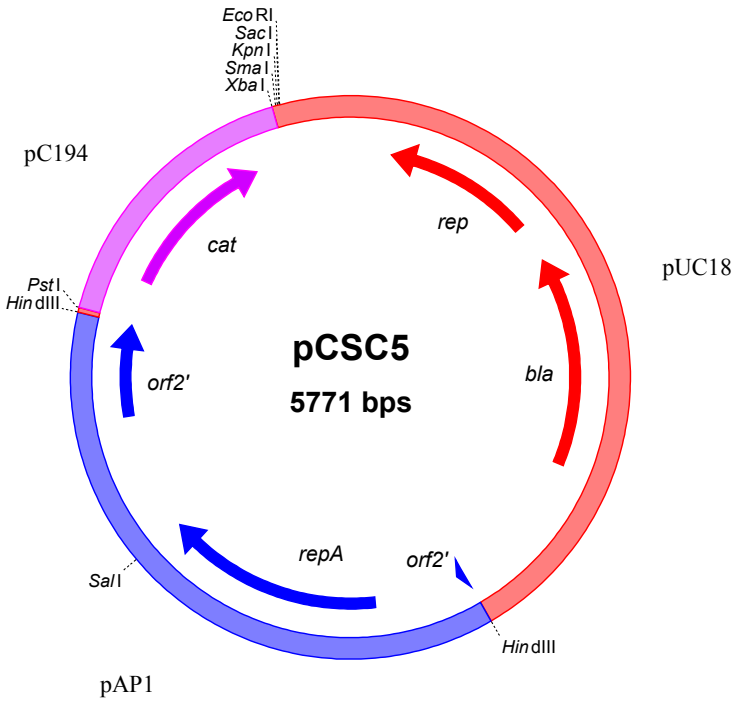


Figure 3-11: Physical map of the plasmid pCSC5. Plasmid pLME201 has been assembled with the amplified *cat* gene from plasmid pC194 at the *Pst*I/*Xba*I restriction site. r*ep*A: putative replicase gene of pAP1; *orf2'*: truncated *orf2* of pAP1, emerged because of the insertion of pUC18; *rep*: replication region of pUC18; *bla*: gene coding for β-lactamase confers resistance to ampicillin; *cat*: gene coding for chloramphenicol-acetyltransferase from pC194, confers resistance to chloramphenicol (Fretz, 2000).

3.4.2 Vectors pCSC3, pCSC4, pCSC6 and pCSC7: derivatives of plasmid pLME202

The *Sal*I linearised pAP1 plasmid from *B. asteroides* DSMZ 20089^T had been cloned into pUC18 at the *Sal*I site. This resulted in plasmid pLME202 (Kaufmann, 1998). Afterwards, the amplified and *Pst*I-digested *tet*(W) gene from pGHost9::*tet*(W) with primer pair cstet/cstet2.rev (table 2-3) was assembled with the pUC18 part of pLME202 at the *Pst*I restriction site. This yielded plasmids pCSC3 and pCSC4. The same strategy was used for the *Pst*I-digested *cat* gene from pC194 and pLME202 yielding pCSC6 and pCSC7. The obtained vectors are shown in figure 3-12 and 3-13. pCSC3 and pCSC4 only differ in the respective orientation of the *tet*(W) gene, in the same way as the orientation of the *cat* gene is opposite in pCSC6 and pCSC7.

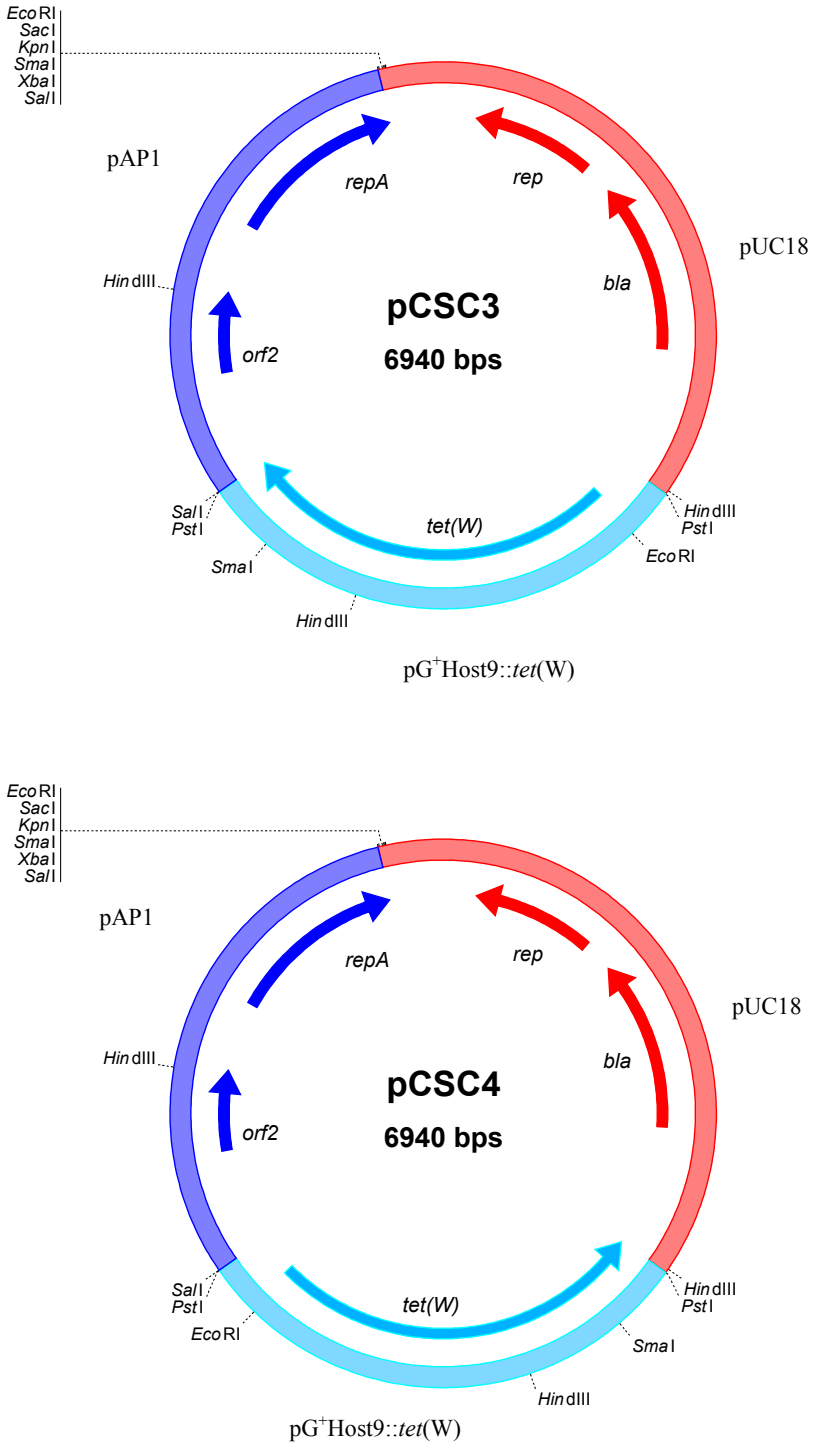


Figure 3-12: Physical maps of the plasmids pCSC3 and pCSC4. The plasmid pLME202 has been assembled with the *tet*(W) gene amplified from plasmid pGHost9::*tet*(W) at the *Pst*I restriction site. *rep*A: putative replicase gene of pAP1; *orf2*: cryptic gene of pAP1; *rep*: replication region of pUC18; *bla*: gene coding for β-lactamase confers resistance to ampicillin; *tet*(W): ribosome-protection tetracycline resistance determinant of *Bifidobacterium* sp.

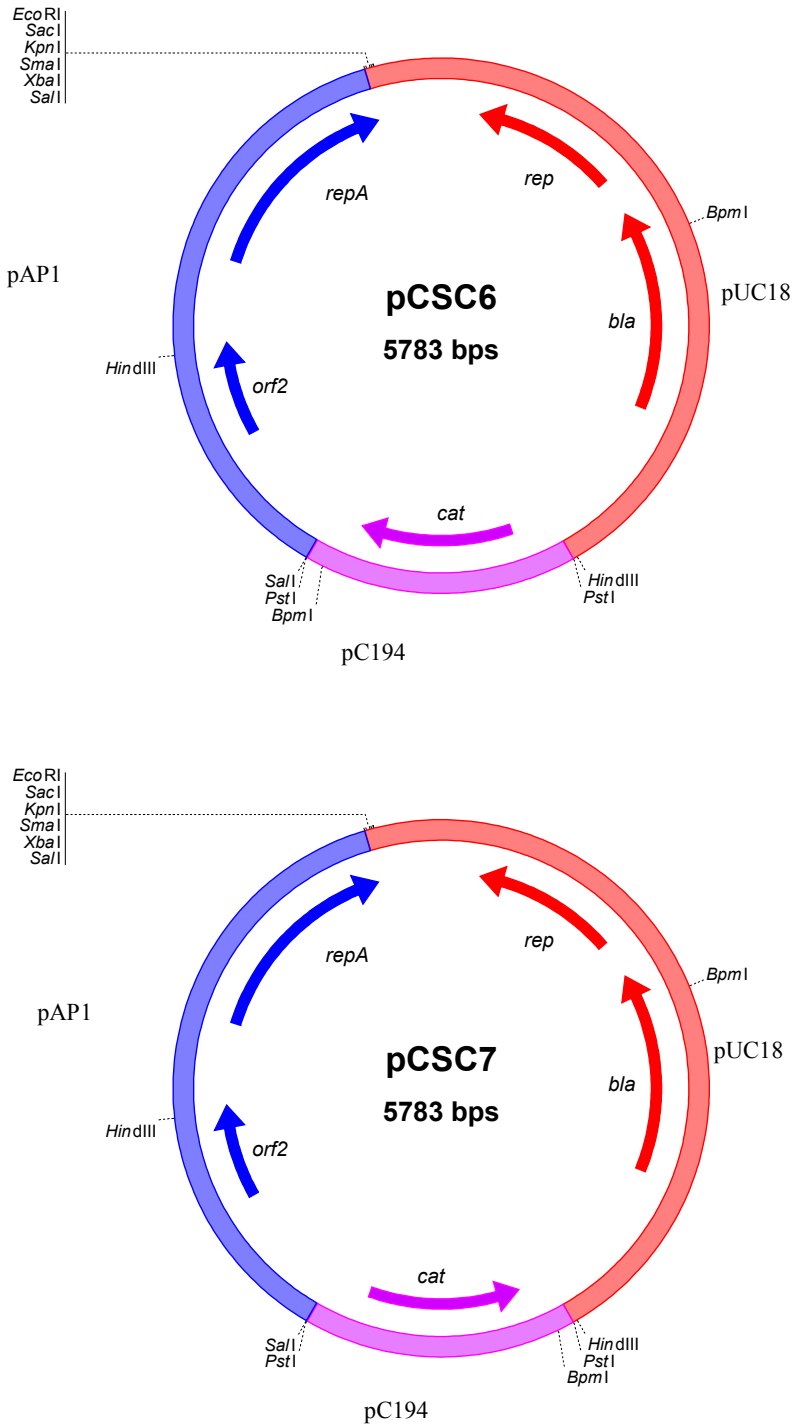
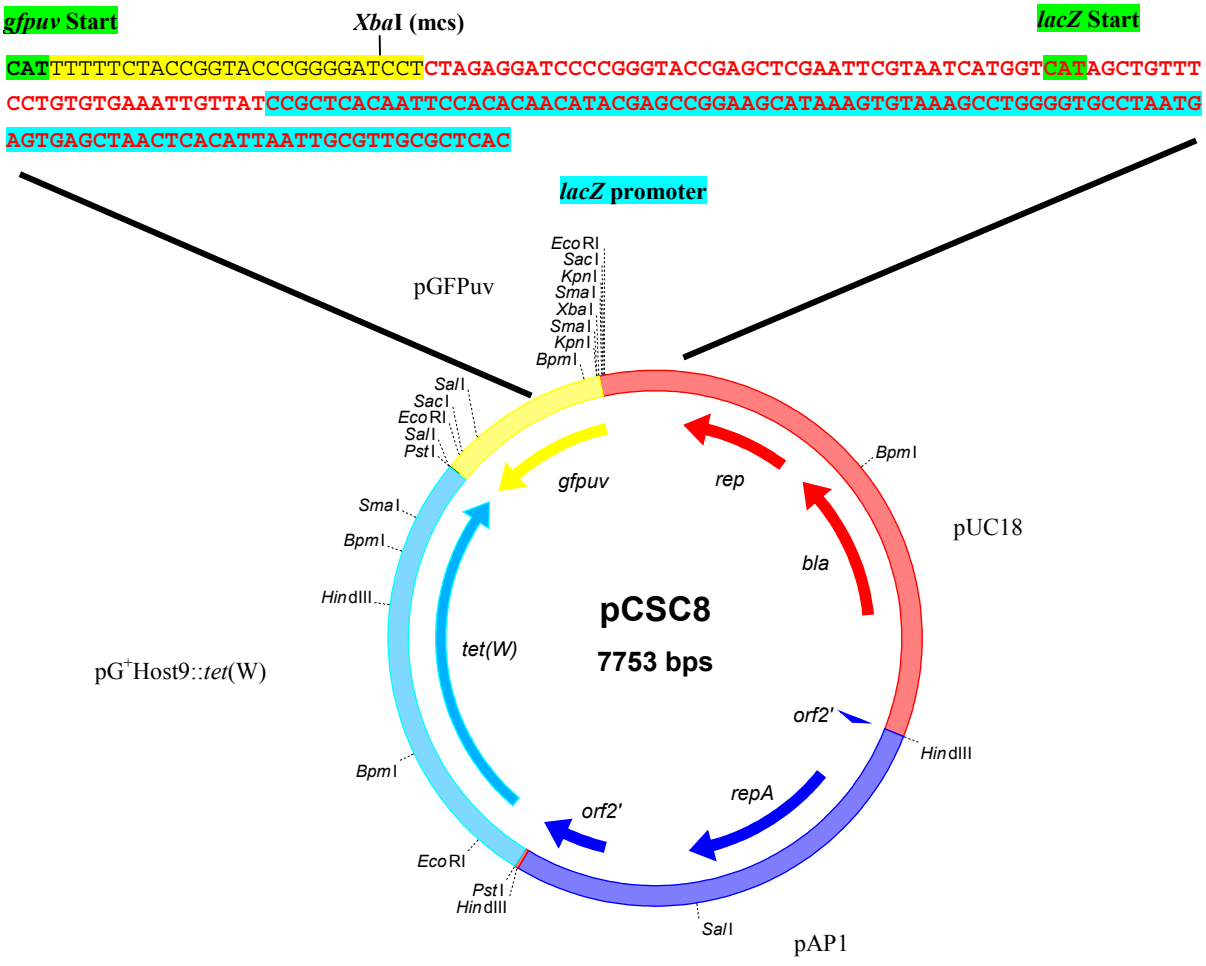


Figure 3-13: Physical maps of the plasmids pCSC6 and pCSC7. The plasmid pLME202 has been assembled with the *cat* gene amplified from plasmid pC194 at the *Pst*I restriction site. *rep*A: putative replicase gene of pAP1; *orf2*: cryptic gene of pAP1; *rep*: replication region of pUC18; *bla*: gene coding for β-lactamase confers resistance to ampicillin; *cat*: gene coding for chloramphenicol-acetyltransferase, confers resistance to chloramphenicol (Fretz, 2000).

3.4.3 Vectors pCSC8 and pCSC9: derivatives of plasmid pCSC1

The two vectors pCSC8 and pCSC9 were constructed for expression experiments. The commercially available plasmid pGFPuv (see table 2-2) contains the complete *gfpuv* gene flanked by two multiple cloning sites and the *lacZ* promoter which regulates gene expression. The promoterless *gfpuv* gene region was excised from plasmid pGFPuv using the restriction enzymes *Xba*I (located within the 5' multiple cloning site of pGFPuv) and *Spe*I (within the 3' multiple cloning site of pGFPuv). Subsequently, this gene region was joined together with the vector pCSC1 at the *Xba*I site directly after the *lacZ* promoter and *lacZ* start codon of the pUC18 part of pCSC1. The resulting plasmids are shown in figure 3-14 and differ only in the orientation of the *gfpuv* gene. The joining region between *gfpuv* and *lacZ* is shown enlarged in figure 3-14. The sequences marked in green are translational start codons either for the *gfpuv* gene (left) or for the *lacZ* gene from pUC18 (right). The blue marked sequence indicates the *lacZ* promoter. The yellow sequence belongs to the *gfpuv* gene and the red one indicates the *lacZ* sequence region.



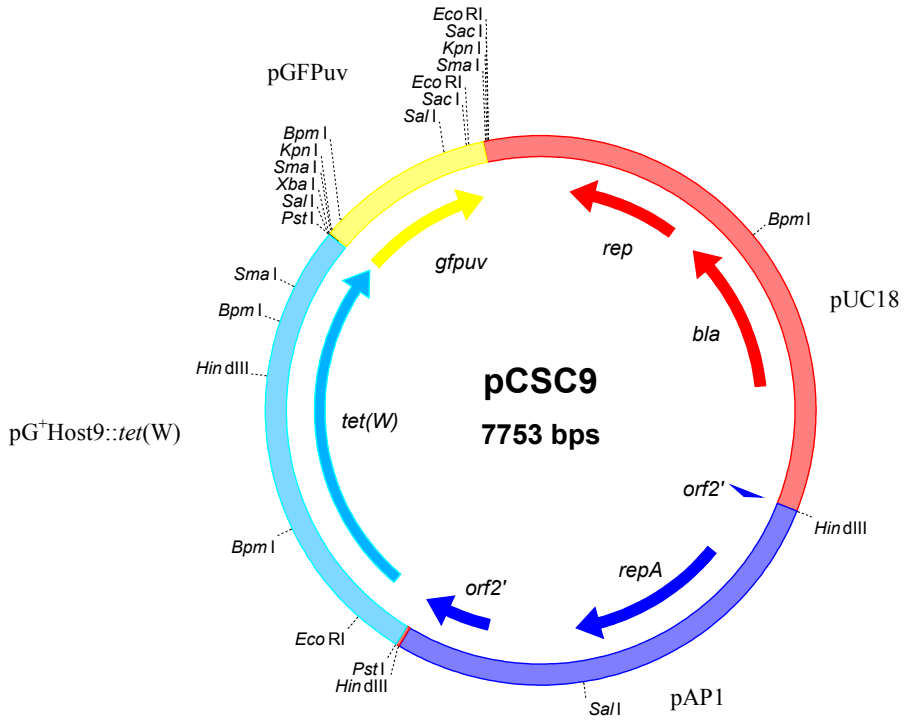


Figure 3-14: Physical maps of the plasmids pCSC8 and pCSC9 with the enlarged transient region of *gfpuv* and *lacZ*: The sequences marked in green are translational start codons either for the *gfpuv* gene (left) or for the *lacZ* gene from pUC18 (right). The blue marked sequence indicates the *lacZ* promoter. The yellow sequence belongs to the *gfpuv* gene and the red one indicates the *lacZ* sequence region. The plasmid pCSC1 has been assembled at the *Xba*I restriction site with the *gfpuv* gene region excised from plasmid pGFPuv using the restriction enzymes *Xba*I and *Spe*I. *rep*A: putative replicase gene of pAP1; *orf2'*: truncated *orf2* of pAP1, emerged because of the insertion of pUC18; *rep*: replication region of pUC18; *bla*: gene coding for β-lactamase confers resistance to ampicillin, *tet*(W): ribosome-protection tetracycline resistance determinant of *Bifidobacterium* sp.; *gfpuv*: gene coding for green fluorescence protein from pGFPuv. Start: translational start codons.

3.5 DNA transformation of *Bifidobacterium*

Transformation experiments with various bifidobacteria were carried out using 8 different methods as listed in table 2-5 and 2-6. Bifidobacteria were always harvested from a liquid culture at an optical density (OD_{600}) of 0.2-0.4 after 1 to 4 hours of incubation (see chapter 2.6.3.1). Transformants were detected on selective solid medium after 3 to 14 days of incubation. As a negative control, cells electroporated without vector DNA were streaked out on the same selective solid medium.

3.5.1 Transformation experiments of *Bifidobacterium* with plasmid DNA isolated from *E. coli* or *Bifidobacterium*

To obtain plasmid DNA in a higher amount the newly constructed vectors had been first introduced into *E. coli* strains. Hence, for the first attempts to transform bifidobacteria, DNA isolated either from *E. coli* XL1-Blue or *E. coli* JM109 was used. The transformation with the DNA isolated from *E. coli* was rather inefficient or didn't work (see table 3-4). This is the reason why such a high number of different transformation methods was used (see chapter 2.6.3). After the first successful introduction of plasmid DNA into bifidobacteria further transformation experiments were made with the reisolated bifidobacterial DNA. The intention was to improve the transformation efficiency in using homologous DNA. During the experiments the *Bifidobacterium-E. coli* shuttle vector plasmid pDRC102 (gift from F. Arrigoni, protected by a patent) served as positive control to check the competence of bifidobacteria cells.

All electrotransformation experiments which resulted in the formation of colonies on selective solid medium are summarised in table 3-4. In case bifidobacterial DNA was used for transformation the plasmid in the table is marked with an asterisk (*). The presence and structure of recombinant plasmids in bifidobacteria transformants were confirmed by small scale plasmid isolation in addition with Southern hybridisation (see figure 3-15 (B)) and by comparison of plasmid size and restriction patterns (see figure 3-15).

In some experiments cell growth on selective solid agar occurred but no plasmid could be isolated. Two possible reasons were suggested for this phenomenon. On one hand, these cells could be spontaneous mutants. On the other hand, the plasmid could be integrated into the chromosome or the small scale plasmid isolation method did not work. Therefore, it was attempted to amplify the selection maker gene region of these cells with the more sensitive PCR method. With an additional Southern hybridisation it was tested if the plasmid had integrated into the chromosome.

Table 3-4: Summary of transformation experiments with *Bifidobacterium* cells grown on selective solid medium using different electroporation methods, vectors, plasmids and strains.

Method[a]	Strain[b]	Vector	Selection marker	DNA amount	Isolation of plasmid	PCR	Number of transformants per µg of DNA
2	*B. lactis* UR1	pCSC1/3	*tet*(W)	1.4 µg	no	no	none
2	*B. angulatum*^{T}	pCSC4	*tet*(W)	0.8µg	no	no	none
4	*B. infantis*^{T}	pCSC7	*cat*	0.8µg	no	yes	none
4	*B. animalis*	pCSC6	*cat*	0.8µg	no	yes	none
5	*B. animalis*	pEC-K18mob2	km^R	0.8µg	no	no	none
5	*B. longum*^{T}	pRDC102	spec^R	0.5µg	no	no	none
6	*B. animalis*^{T}	pRDC102	spec^R	0.5µg	yes	nt	20
6	*B. longum* NCC 2705	pRDC102	spec^R	0.5µg	yes	nt	80-130
7	*B. longum*	pCSC6/4	*cat/tet*(W)	0.5µg	no	no	none
	NCC 2705	pRDC102	spec^R	0.5µg	yes	nt	110-140
		pCSC1	*tet*(W)	0.5µg	yes	nt	3-5
		pCSC1*	*tet*(W)	0.8µg	yes	nt	25-30
7	*B. animalis*	pCSC1	*tet*(W)	0.5µg	no	nt	none
		pRDC102	spec^R	0.5µg	no	nt	none
7	*B. animalis*^{T}	pCSC1	*tet*(W)	0.5µg	no	nt	none
7	*B. asteroides*	pCSC1/2/3/4	*tet*(W)	0.5µg	no	nt	none
		pRDC102	spec^R	0.5µg	no	nt	none
8	*B. longum*	pRDC102	spec^R	0.5µg	no	nt	none
	NCC 2705	pCSC1*	*tet*(W)	0.8µg	yes	nt	300-320

[a] Details to the methods are described in chapter 2.6.3, tables 2-5 and 2-6

[b] All strains used are listed in table 2-1, ^{T} indicates a DSMZ type strain

***** Indicates that plasmid DNA was isolated from bifidobacteria

nt: not tested

The shaded lines indicate successful transformation experiments

The highlighted experiments in the table show successful transformation experiments. The experiments with the control plasmid pRDC102 resulted in *B. animalis*^{T} and *B. longum* NCC 2705 transformants with an efficiency of 20 to 140 transformants/µg DNA depending on the chosen method. With plasmid pCSC1 only *B. longum* NCC 2705 transformants were obtained.

The transformation efficiency ranged from 3-5 transformants/µg DNA, if the transformed DNA was isolated from *E. coli*, to 300 transformants/µg DNA, if bifidobacterial DNA was used for transformation. As one example figure 3-15 shows the confirmation of a *B. longum* NCC 2705 transformant harbouring plasmid pCSC1, including small scale plasmid isolation (picture A), Southern hybridisation (picture B) with the [α-^{32}P]-labelled pCSC1 and the restriction patterns of the recombinant plasmid (picture C). The supercoiled DNA ladder (sc-ladder) hybridised with pCSC1 because the DNA of this marker contains parts of pUC18. With these positive results the existence of pCSC1 in the *B. longum* NCC 2705 transformants was proven.

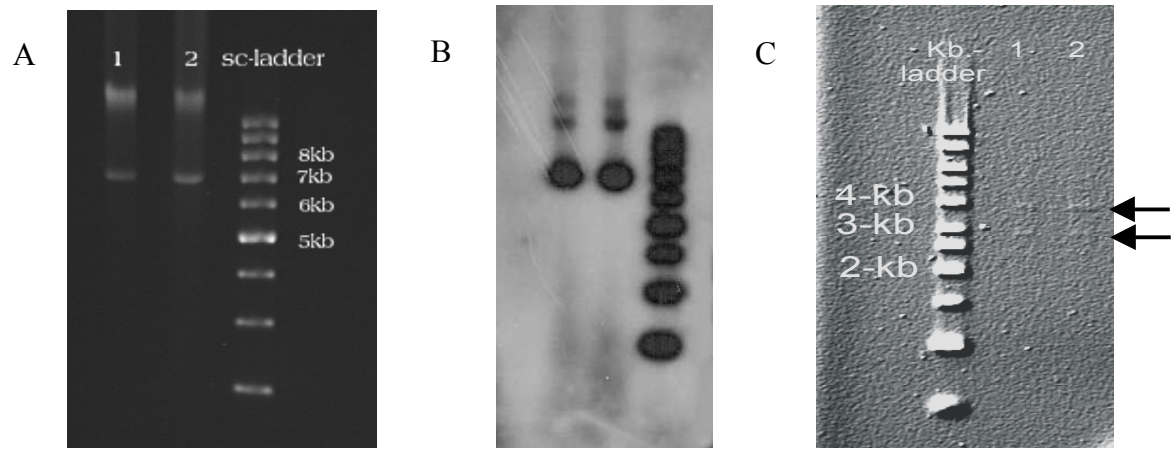


Figure 3-15: Confirmation of recombinant plasmids from the transformed *B. longum* NCC 2705 with the 6.9-kb plasmid pCSC1, containing *tet*(W) as marker. A: Small scale plasmid isolation of a *B. longum* colony grown on selective medium (lane 1) and the pCSC1 plasmid as positive control (lane 2). B: Southern hybridisation with [α-^{32}P]-labelled pCSC1 isolated from *E. coli* XL1-Blue as probe. C: Restriction patterns of the recombinant plasmids digested with the enzyme *Sal*I. Arrows indicate position of plasmid DNA bands.

Stierli (2002) transformed propionibacteria in her work. She showed that the transformation efficiency could be a 1000 fold increased by using unmethylated DNA. Therefore, it was tested whether bifidobacteria would take up unmethylated DNA from *E. coli* JM110 with higher efficiency than the methylated DNA from *E. coli* XL1-Blue in the same way as propionibacteria do. *B. longum* NCC 2705 was transformed with the control vector pRDC102 reisolated from *E. coli* JM110: no increased transformation efficiency was obtained (data not shown).

All other transformation experiments listed in table 3-4 resulted in appearance of colonies on selective solid medium, but no plasmids could be isolated. In some cases it was possible to amplify the antibiotic selection marker gene of the used vector (see table 3-4). The additional hybridisation, using either the [α-^{32}P]-labelled plasmid or the antibiotic resistance gene as probe gave no signal for the isolated plasmid, but a positive signal was obtained for the amplified antibiotic resistance gene (data not shown). For further explanations of this phenomenon see chapter 4.2.5.

The residual experiments, which are not listed in table 3-4, failed and no growth could be detected on selective medium.

3.5.1.1 Effect of pCSC1 DNA concentration, isolated from *B. longum* NCC 2705, on transformation efficiency of *B. longum* NCC 2705

B. longum NCC 2705 was transformed according to method 7 with different amounts of plasmid pCSC1 DNA. This DNA was isolated from a recombinant *B. longum* NCC 2705 strain. The transformation efficiency yielded with various DNA amounts is shown in figure 3-16. Between 200 and 314 transformants were obtained per µg DNA. Obviously, the number of transformants/µg DNA did not increase linearly with the DNA amount and with DNA amounts higher than 0.8µg even a decreased transformation rate was observed.

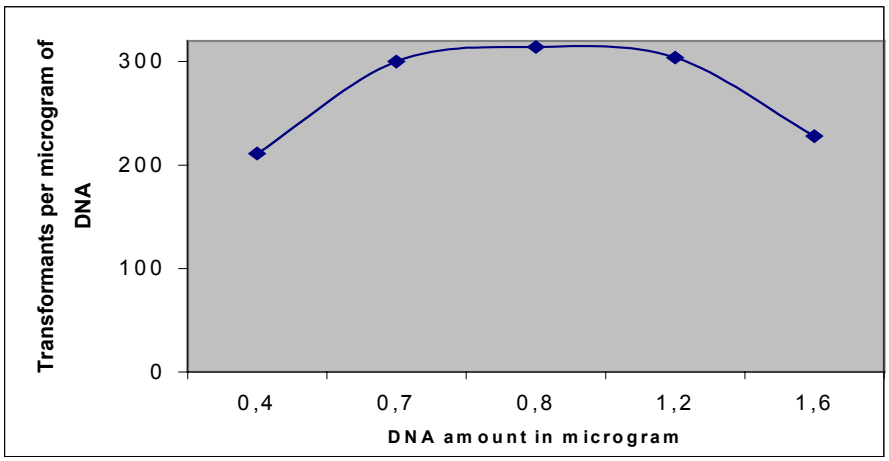


Figure 3-16: *B. longum* NCC 2705 transformation rate per µg DNA of plasmid pCSC1 isolated from recombinant *B. longum* NCC 2705.

3.5.1.2 Segregational stability of *Bifidobacterium-E. coli* shuttle vector pCSC1

B. longum NCC 2705 transformants containing pCSC1 were cultivated in liquid MRS medium lacking tetracycline. As a control, the same transformants were cultivated in selective liquid MRS medium containing 10µg/ml tetracycline. Subsequently, the cultures were streaked out on MRS agar plates containing 10µg/ml tetracycline. After 8 generations $7x10^7$ colony forming units per ml could be detected on selective agar plates, while on the control plates $24x10^7$ cfu/ml grew. After 14 generations the resistant colonies decreased under the level of 10^2 colony forming units per ml.
The structural stability of the vector plasmids was studied by cultivation of transformants in selective liquid medium for about 20 generations. *B. longum* NCC 2705 transformants which harboured pCSC1 or pRDC102 showed no decrease of their growth rate. Plasmids isolated from these transformants showed the same size and restriction patterns as the original plasmids (data not shown).

3.5.1.3 Minimal inhibitory concentration (MIC) of antibiotics of *E. coli* and *Bifidobacterium* transformants

To verify the expression of the antibiotic resistance genes of the newly constructed vectors, the transformants' minimal inhibitory concentrations of chloramphenicol and tetracycline were determined using "Etest" stripes.
All *E. coli* XL1-Blue and *E. coli* JM109 transformants which harboured the plasmids pCSC5, pCSC6 and pCSC7 were assayed for their minimal inhibitory concentration of chloramphenicol. Due to the intrinsic tetracycline resistance of *E. coli* XL1-Blue, only *E. coli* JM109, carrying pCSC1, pCSC2, pCSC3 or pCSC4, was tested for tetracycline susceptibility. Furthermore, the recombinant *B. longum* NCC 2705 strain in which pCSC1 had been inserted was examined for tetracycline susceptibility as well.
The recipient strains without plasmids were also tested for comparison. The results of the "Etests" are listed in table 3-5.

Table 3-5: Minimal inhibitory concentration of chloramphenicol and tetracycline of *Bifidobacterium* and *E. coli* transformants harbouring different vectors, and of the recipient strains without plasmids, determined using "Etest" stripes.

Recipient strain	**MIC's[a] (µg/ml) of tetracycline of the recipient containing:**				
	pCSC1	pCSC2	pCSC3	pCSC4	no plasmid
E. coli XL1-Blue	ir	ir	ir	ir	ir
E. coli JM109	8	8	12	12	1
B. longum NCC 2705	12	-	-	-	0.125

Recipient strain	**MIC's[a] (µg/ml) of chloramphenicol of the recipient containing:**			
	pCSC5	pCSC6	pCSC7	no plasmid
E. coli XL1-Blue	>256	>256	>256	3
E. coli JM109	>256	>256	>256	2
B. longum NCC 2705	-	-	-	0.38

[a] MIC: minimal inhibitory concentration according to the "Etest"
ir: intrinsically resistant

Obviously, the chloramphenicol-acetyltransferase gene of the newly constructed vectors pCSC5, pCSC6 and pCSC7 was well expressed in both *E. coli* strains and 100 fold improved MIC's were measured. The tetracycline resistance gene of pCSC1, pCSC2, pCSC3 and pCSC4 increased the MIC of *E. coli* JM109 about 10 fold. Plasmid pCSC1 which was transformed in *B. longum* NCC 2705 could effect an 100 fold increase of the MIC of tetracycline. Obviously the transformant could express the *tet*(W) gene. Additionally, identical MIC's were measured for plasmids which contain the resistance marker gene in opposite reading direction. These results indicate that the *E. coli* RNA-polymerase recognised the *tet*(W) as well as the *cat* promoter. Due to the fact that *B. longum* NCC 2705 transformants could only be obtained with pCSC1 but none containing pCSC2, which only differs from pCSC1 in the orientation of the *tet*(W) gene, it must be assumed that *tet*(W) is expressed in *B. longum* by a promoter which is encoded on pAP1.

3.5.2 Transformation of *E. coli* XL1-Blue with pCSC1 and pRDC102 DNA reisolated from *Bifidobacterium* transformants

In order to prove the suitability of the constructed vector as *Bifidobacterium-E. coli* shuttle vector, *E. coli* XL1-Blue cells were transformed with DNA reisolated from *B. longum* NCC 2705 transformants. Numerous transformants of *E. coli* were obtained on selective agar plates containing 60µg/ml ampicillin. Successful transformation was confirmed by small scale plasmid isolation and comparison of restriction patterns with the original plasmids pCSC1 or pRDC102 (data not shown).

3.5.3 Transformation of *B. longum* NCC 2705 with the reporter vectors pCSC8 and pCSC9

The *B. longum* NCC 2705 transformant carrying vector pCSC1 could express the homologous tetracycline resistance gene *tet*(W). In a following experiment, the expression ability of a heterologous gene in bifidobacteria was tested with two new vectors. The reporter vectors pCSC8 and pCSC9 contained plasmid pCSC1 and the promoterless green fluorescence protein gene *gpfuv* in opposite reading directions (see chapter 3.4.3). The transformation experiments were set up according to methods 7 and 8 (see chapter 2.6.3). The plasmid DNA of pCSC8 and pCSC9 used for transformation was obtained from a large scale plasmid isolation of *E. coli* XL1-Blue. Only one transformant was obtained with the 7.7-kb plasmid pCSC8, named CSC8. Its small scale plasmid isolation is shown in figure 3-17, lane 4.
This transformant grew on selective agar containing 10µg/ml tetracycline after one week of anaerobic incubation at 37°C. In liquid media, also supplemented with 10µg/ml tetracycline, CSC8 grew within 3 days at 37°C. To test the expression of the green fluorescent protein GFPuv, CSC8 was plated on solid selective medium and was incubated either at 37°C or at 30°C. But after 2 days of incubation no fluorescence emission was detectable under UV light (302nm). Afterwards, the colonies were aerobically stored at room temperature overnight and tested for fluorescence again, but still in vain. The *E. coli* XL1-Blue transformant carrying pCSC8 served as control for fluorescence emission of the GFPuv.

The segregational stability of the plasmid was very low. After 2 fresh inoculations of CSC8, incubated overnight in liquid medium containing 10µg/ml tetracycline, the plasmid could not be detected anymore. The loss of the plasmid and the inability to express the *gfpuv* gene of the plasmid could be a result of problems concerning replication, transcription or translation caused by the lack of a suitable promoter or the plasmid size itself.

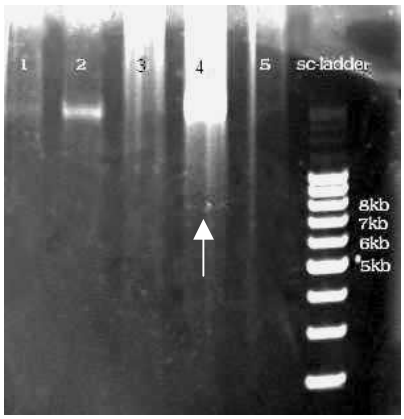


Figure 3-17: Small scale plasmid isolation of 4 transformed *B. longum* NCC 2705 strains with the 7.7-kb vector pCSC8. The only transformed plasmid is visible as a faint band in lane 4.

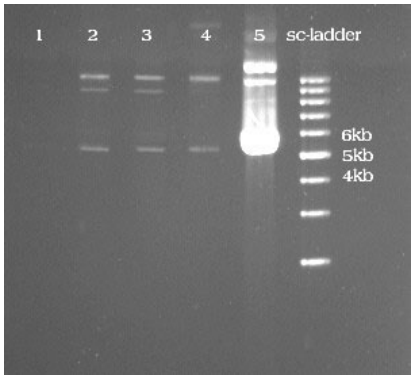
3.5.4 Transformation of *Bifidobacterium* with the *Corynebacterium-E. coli* shuttle vector pEC-K18mob2

The group of Argnani *et al.* (1996) demonstrated the ability of two *Corynebacterium* plasmids to transform *B. animalis* DSMZ 20105. Hence, we attempted to transform bifidobacteria with the *Corynebacterium-E. coli* shuttle vector pEC-K18mob2 (see table 2-2.). *B. infantis* DSMZ 20088^T and *B. animalis* DSMZ 20105 were transformed as described in method 6 (see chapter 2.6.3). Transformed cells were selected on MRS solid medium containing 25µg/ml kanamycin, but no transformants were ever obtained (Hoppler, 2001).

3.6 Transformation of *Corynebacterium glutamicum* ATCC 13032 with plasmids pCSC6 and pCSC7

Hoppler (2001) tested in his diplomawork the suitability of the bifidobacterial replicon of the constructed vectors with an established transformation system of a phylogenetically related bacteria. For this purpose the Gram-positive, high G+C bacteria *Corynebacterium glutamicum* ATCC 13032 was used. The transformation experiments were performed with the plasmid constructs pCSC6 and pCSC7, conferring chloramphenicol resistance. The *Corynebacterium-E. coli* shuttle vector pEC-K18mob2 served as a positive control for successful transformation. Recombinant *C. glutamicum* were selected on *Corynebacterium* agar supplemented with 5µg/ml chloramphenicol. They were confirmed with a small scale plasmid isolation, and a Southern hybridisation with the [α-^{32}P]-labelled plasmid pLME202 as a probe (figure 3-18). The small scale plasmid isolations and hybridisation signals of pCSC6 from *Corynebacterium* transformants were visible in lanes 1 and 2; the isolations of pCSC7 are shown in lanes 3 and 4. The supercoiled DNA ladder (supercoiled sc-ladder) hybridised with pLME202 (figure 3-18 (B), last lane) because the DNA of this marker contains parts of pUC18. Additionally, a PCR with primer pair cscat2/cscat3.rev (see table 2-3) was performed with the transformants to amplify the *cat* gene (950bp) from pCSC6 and pCSC7. The faint PCR fragments are shown in figure 3-19 (lanes 1 to 10). The PCR fragments shown in the lanes 1, 2, 4 and 6 were obtained with the corresponding transformants whose plasmid isolations are shown in figure 3-18, lanes 1-4.

A



B

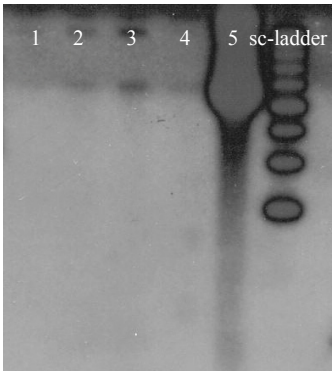


Figure 3-18: Confirmation of recombinant plasmids from the transformation of *C. glutamicum* ATCC 13032 with plasmids pCSC6 and pCSC7. A: Lanes 1-2: Small scale plasmid isolation of transformed *C. glutamicum* with plasmid pCSC6. Lanes 3-4: Small scale plasmid isolation of transformed *C. glutamicum* with plasmid pCSC7. Lane 5: pCSC6 isolated from *E. coli* XL1-Blue. B: Southern hybridisation with [α-^{32}P]-labelled pLME202 isolated from *E. coli* XL1-Blue as probe.

Figure 3-19: Amplification of the *cat* gene (950bp) with the primer pair cscat2/cscat3.rev from recombinant *C. glutamicum* ATCC 13032 transformed with pCSC6 and pCSC7. Lanes 1-10: Amplified *cat* gene of transformants. Lane 11: positive control, *cat* amplified from pC194.

The transformation efficiency of the plasmids pCSC6 and pCSC7, which was obtained with plasmid DNA isolated from *E. coli* XL1-Blue, was 13 to 22 transformants/µg DNA. The transformation rate was therefore slightly higher than that one of pCSC1 transforming *B. longum* NCC 2705. Even though, the purpose of this experiment to prove the replication ability of the constructed plasmids was achieved. *C. glutamicum* transformants grew on agar plates with a chloramphenicol concentration of 5µg/ml but not with 10µg/ml. Therefore, it was concluded that the chloramphenicol-acetyltransferase gene could be expressed but at a low level. Due to the low transformation efficiency the plasmids pCSC6 and pCSC7 are not dedicated as *Corynebacterium-E. coli* vectors for routine transformation experiments.

4 Discussion

4.1 Specifity of a new PCR approach to identify *Bifidobacterium*

Due to the non-sterile atmosphere in the anaerobic chamber, bifidobacteria cultures had to be routinely controlled during this work. Therefore, a PCR strategy was designed to detect contaminated bifidobacteria cultures. This strategy was based on 16S rRNA differences and had to be tested for its practical utility and its specificity for the genus *Bifidobacterium* relying on 16S rRNA GenEMBL database information.

The strategy was founded on a unique 16S rRNA gene sequence region of the genus *Bifidobacterium,* representing the target for either primer bak11w or lm26. The two primers do not only differ in their starting position (nucleotide 8 for bak11w and 15 for lm26 with respect to the *E. coli* 16S rRNA gene (Brosius *et al.*, 1978)). Primer bak11w also contains the nucleotides CM at position 16 and 17 instead of TC at the corresponding position in lm26.

These differences in primer sequences are the reason why bak11w, regarded as universal for bacterial 16S rRNA (Goldenberger, 1997), should not anneal with bifidobacterial 16S rRNA. Primer lm26 in contrast was especially designed for the determination of bifidobacteria (Kaufmann *et al.*, 1997b).

To check culture purity two PCR approaches were made. One approach used primer pair lm26/lm3 which amplified a specific 1.35-kb fragment from bifidobacteria. In the second PCR approach, primer pair bak4/bak11w produced a 1.4-kb fragment from all other bacteria. If the culture only contained bifidobacteria, one single fragment with primer pair lm26/lm3 was amplified. If the culture was contaminated, both primer pairs amplified fragments.

To prove the functionality of this strategy, 16S rRNA GenEMBL database sequences of bifidobacteria were compared with the primer sequences of bak11w and lm26. The alignment in figure 3.1 shows that 33 out of 42 bifidobacterial 16S rRNA sequences contained 1 or more mismatches to bak11w, but showed 100% identity to the sequence of primer lm26.

From the 9 remaining sequences the sequence of *B. bifidum* contained several unidentified nucleotides (N) at the relevant region. The sequences of *B. dentium* and *B. pullorum* had been submitted to the GenEMBL database by the group of Watanabe.

For all 3 strains the PCR test gave no signal for primer pair bak11w/bak4 but for primer pair lm3/lm26 a distinct signal was visible (shown in figure 3-2, lanes 17/18 for *B. dentium*). For the strains *B. indicum*, *B. infantis*, *B. magnum* and *B. globosum* two different sequences exist in the database. Always one of these two sequences was submitted by Watanabe and contained no mismatches to bak11w, the other sequences of the same strains showed at least 3 mismatches. The PCR with these strains gave no signal using primer pair bak11w/bak4 but a distinct signal for lm3/lm26.

The *B. inopinatum* type strain sequence also exists twice in the GenEMBL database (see figure 3-1). One of these sequences was submitted by the group of Watanabe and contained 3 mismatches. The group of Dong sequenced the other which did not contain any mismatches to primer bak11w. In the experiments made with *B. inopinatum*, fragments were amplified with primer pair bak11w/bak4, but with primer pair lm3/lm26 no fragments were obtained. The distinctly low DNA G+C content of *B. inopinatum* might have caused its special sequence, which differs from all other *Bifidobacterium* strains.

The last sequence which did not contain any mismatches to primer bak11w was from *B. thermacidophilum,* sequenced by the group of Dong. Due to the only recent isolation of *B. thermacidophilum*, no DSMZ type strain was commercially available, and no further statements could be made about this strain.

As a consequence, the PCR strategy using the two primer pairs bak11w/bak4 to amplify the 16S rRNA gene fragment of possible contaminants and lm3/lm26 for the amplification of the 16S rRNA gene fragment of bifidobacteria species seems to be a simple and fast method to check the purity of *Bifidobacterium* isolates and cultures.

4.1.1 Limited usability of selective media for *Bifidobacterium* isolation

The four different selective media Columbia broth, RB broth, BHI + mupirocin broth and BFM broth (see chapter 2.1), were tested for their effectiveness to discriminate *Bifidobacterium* from *Enterococcus* strains. All these media had been developed originally to enumerate bifidobacteria and separate them from other lactic acid bacteria in dairy products. However, in our work most contaminant bacteria found in bifidobacteria cultures came from experimental handling and methods. It turned out that they were mostly enterococci as determined by partial 16S rRNA gene sequencing (data not shown).

In our experiments, contaminated cultures (determined by PCR) were inoculated into the above mentioned selective media and single colonies were checked afterwards by PCR again (see figures 3-2 to 3-5). None of the tested media showed increased selectivity for bifidobacteria compared to the routinely used BHI or MRS medium, but all these selective media were difficult and time consuming to prepare. Thus, the problem to separate bifidobacteria from enterococci could not be solved with such a simple method as selective differentiation media since these two bacteria grew well under the same incubation conditions like pH-value, temperature and nutrition requirements (Fretz, 2000). This is not surprising, as the natural habitat of both of the bacteria is the human or animal intestine.

Several studies have compared selective bifidobacteria media, but almost every study was restricted to the differentiation between bifidobacteria and lactic acid bacteria. In the most extensive study, over 20 selective media were tested to separate *Bifidobacterium* from *Lactobacillus* in dairy products (Charteris *et al*., 1997). In one study only, four selective media were tested for their functionality in isolating faecal bifidobacteria (Martineau, 1999). It turned out that the BS-MCA medium, containing neomycin sulphate, propionic acid, lithium chloride and paromycin sulphate, showed best results, but *Bifidobacterium* cells had a low viability. All other media were not suitable for selection and isolation of bifidobacteria from faecal samples. These findings correspond with my observations.

4.2 Development of a transformation system for *Bifidobacterium*

The endeavour of this thesis was the development of a transformation system for *Bifidobacterium*. Different research groups presented *Bifidobacterium-E. coli* shuttle vectors. They used a *Bifidobacterium* plasmid and assembled it with commercial available *E. coli* vector plasmids. As antibiotic selection markers the chloramphenicol-acetyltransferase gene of plasmid pC194, or spectinomycin or erythromycin resistance genes from *Enterococcus faecalis* and *Staphylococcus aureus,* respectively were employed. Consequently, this strategy was used to construct a new *Bifidobacterium-E. coli* shuttle vector in this study.

4.2.1 Inefficient plasmid screening from human faecal *Bifidobacterium*

The intention was to find an endogenous plasmid from a human intestinal *Bifidobacterium* which was suitable for shuttle vector construction.
Bifidobacterium strains from human faecal samples grew on BHI agar plates under anaerobic conditions. They were identified by colony hybridisation with the [γ-^{32}P]-labelled probe lm3 and were confirmed by PCR. From 700 isolated strains only 3.5%, which means 24 strains, could be verified as bifidobacteria (see table 3-2). These isolates were screened for plasmid contents. Only large plasmids were found. Their sizes ranged between 8- and over 10kb. It was considered that these plasmids are too large to be used directly for vector constructions (see table 3-3) without time consuming sequencing experiments of the plasmid or deletion experiments to narrow down on the essential replication functions.
Caused by the absence of a convenient endogenous plasmid, the already characterised plasmid pAP1 from *B. asteroides* (Kaufmann, 1998) was chosen as basis for vector construction, although *B. asteroides* was originally isolated from a honeybee and not from a human sample.

4.2.2 The choice of chloramphenicol and tetracycline resistance genes as selection markers

After the selection of the plasmid pAP1 as the origin of the new *Bifidobacterium-E. coli* shuttle vector, a suitable selection marker gene was needed. Some groups (Argnani *et al.* 1996, Rossi *et al.* 1996, and Matteuzzi *et al.* 1990) had established *Bifidobacterium-E. coli* transformation systems using the chloramphenicol-acetyltransferase gene from *Staphylococcus aureus* plasmid pC194 as selection marker gene. Consequently, this chloramphenicol-acetyltransferase gene was also applied as a selection marker in this study.
Looking for a second selection marker, the endogenous tetracycline resistance *tet*(W) was chosen. This gene was originally isolated from a *Bifidobacterium* genome (see table 1-2) and had not been used before as selection marker.
For that reason, the minimal inhibitory concentrations of chloramphenicol and tetracycline of all *Bifidobacterium* strains, listed in table 2-1, were determined. The intention was to find sensitive recipient strains for selection in future transformation experiments.

For the determination of the minimal inhibitory concentrations (MIC's) with the "Etests", the softagar overlay method was employed. The growth medium used was MRS and not a standard medium as recommended by the supplier because the MRS medium was also used in the subsequent transformation experiments.

29 *Bifidobacterium* strains were tested as shown in table 3-4. 25 strains showed a minimal inhibitory concentration for both antibiotics of less than 10µg/ml. In consequence these 25 strains were suitable for selection on these two antibiotics after transformation. But due to poor growth rates of 8 of these 25 strains, only 17 strains were used for transformations. Although no standard measuring methods were used in this study, the measured MIC's of 0.047-2µg/ml for chloramphenicol and 0.064-128µg/ml for tetracycline correspond well with the findings by the group of Lim *et al.* (1993). Their attempts described variable minimal inhibitory concentrations from 1.56-6.25µg/ml for chloramphenicol and 0.39-50µg/ml for tetracycline, respectively. Yazid *et al.* (2000) reported that almost every *Bifidobacterium* strain tested was sensitive to chloramphenicol and tetracycline at an antibiotic concentration of 30µg/ml.

4.2.3 Functional selection markers of *Bifidobacterium-E. coli* shuttle vectors in *E. coli*

In this study, several new *Bifidobacterium-E. coli* shuttle vectors have been constructed. The origins of all of these new vectors were the two plasmids pLME201 and pLME202, described by Kaufmann (1998). These two plasmids descended from the *B. asteroides* plasmid pAP1, assembled with the well-known *E. coli* vector plasmid pUC18 at the restriction sites *Hin*dIII (pLME201) and *Sal*I (pLME202). While the *Hin*dIII site of pAP1 is located within the region of the putative gene *orf*2, the *Sal*I site is downstream the putative *repA* gene of the plasmid. To find out whether the *orf2* gene is essential for replication of the plasmid or not, both vectors pLME201 and pLME202 were used for vector constructions.

With these prerequisites seven plasmids have been constructed: pCSC1 and pCSC2 are assemblies of pLME201 and the *tet*(W) gene as selection marker; pCSC3 and pCSC4 of pLME202 and the *tet*(W) as marker gene; pCSC5 of pLME201 and pCSC6 as well as pCSC7 of pLME202 and the *cat* gene as a selection marker (see figures 3-10 to 3-14).

For the construction of these seven plasmids, *E. coli* XL1-Blue and *E. coli* JM109 served as host strains. *E. coli* JM109 was used to circumvent the possible interference of the intrinsic tetracycline resistance of *E. coli* XL1-Blue and the antibiotic resistance genes integrated into the plasmids. The expression of the antibiotic resistance genes in *E. coli* was tested with "Etest" stripes which determined the minimal inhibitory concentrations of the respective antibiotics. Identical MIC's were measured for identical plasmids, carrying the selection marker genes in opposite reading directions. This gives evidence that the promoter of the *tet*(W) gene as well as that of the *cat* gene were recognised in *E. coli*. The MIC's for tetracycline were about 10 fold increased, the MIC's for chloramphenicol about 100 fold compared to the original *E. coli* strain without plasmid (see table 3-5). One of the possible reasons for the different expression of the two genes could be due to their distinct origin. The chloramphenicol-acetyltransferase gene derived from the *Staphylococcus aureus* plasmid pC194 and was already successfully tested for expression in *E. coli* strains (Goze and Ehrlich, 1980), but the *tet*(W) gene had been isolated from chromosomal DNA of a *Bifidobacterium* strain and was never used before in *E. coli*.

4.2.4 Competent cells and transformation conditions

The electroporation conditions for the appropriate method ranged from 10 to 12.5kV/cm and from 100 to 200Ω at 25μF suggested by different authors. All used methods are listed in table 2-5 and 2-6.

In this study two different methods were successfully used for transformation experiments. These two methods were modified methods of Argnani *et al.* (1996) with MRS + 2% glucose or Iwata medium as growth medium, 1mM ammonium citrate buffer with 0.5M sucrose [pH 6.0] as washing and electroporation buffer and preincubation time of 0.5h at 4°C before electroporation with 200Ω, 25μF and 10kV/cm (method 6 and 7 in table 2-5 and 2-6). Method 8 after Rossi *et al.* (2000b) was also successful (see table 3-5).

4.2.5 Host strains and vector plasmids

The preferred host strain of the Argnani and Rossi group was *B. animalis* DSMZ 20105. In our study, 17 different bifidobacteria strains were tested as potential host strains. Finally, successful transformations were only obtained with the *B. animalis* type strain and the *B. longum* NCC 2705 strain, transformed either with control plasmid pRDC102 or with vector plasmid pCSC1, conferring tetracycline resistance.

In the following experiments the *B. longum* NCC 2705 strain and the plasmid pCSC1 served as references to test other strains and plasmids for their electroporation performance.

The *B. longum* NCC 2705 strain was used to be transformed by all constructed vector plasmids and the *E. coli-Propionibacterium* shuttle vector pAMT1 (Stierli, 2002) too, but only pCSC1 was successfully transforming. This led to the following interpretations:

The plasmids pCSC1, pCSC2 and pCSC5 are identical, except for the resistance marker genes. While pCSC1 and pCSC2 carry the *tet*(W) gene in opposite orientations, plasmid pCSC5 contains the *cat* gene as resistance marker. From these three plasmids only pCSC1 transformed *B. longum* NCC 2705. The fact that pCSC2 did not work gives evidence that the promoter of the *tet*(W) gene could not be recognised by *B. longum* NCC 2705 and that the expression of the gene was controlled by a promoter of pAP1 which is only in pCSC1 in the same reading direction and upstream the *tet*(W) gene. Plasmid pCSC5 carries the *cat* gene in the same reading direction as pCSC1 carries the *tet*(W) gene. Rossi *et al.* (1998) demonstrated the capability of bifidobacteria to express exactly the same chloramphenicol-acetyltransferase gene as used in this study. Even though, pCSC5 was not transforming *B. longum* NCC 2705. Therefore, it must be supposed that the expression of the *cat* gene of plasmid pCSC5 could not be induced by a pAP1 promoter. This is perhaps caused by the *Staphylococcus aureus* origin of the gene instead of the *Bifidobacterium* sp. origin of the *tet*(W) gene.

The parental plasmid of pCSC1 was pLME201 which consists of pAP1 and pUC18 assembled at the *Hin*dIII site. Since this restriction site is located within *orf2*, a putatively translated function of this gene could not be involved in replication mechanisms of the plasmid.

All the transformation experiments, according to reference method 7, with plasmid pCSC1 using the *B. animalis* strain from Argnani, the *B. animalis* type strain or the *B. asteroides* DSMZ 200431 strain, failed. The reason for the unsuccessful transformation of *B. asteroides* could be the poor growth of this strain.

Amazingly, none of the *B. animalis* strains could be transformed although Argnani *et al.* (1996) and Rossi *et al.* (1998) showed the transformation ability of *B. animalis* DSMZ 20105. Since a faint growth of the electroporated *B. animalis* strains on selective agar plates was observed, it was assumed that these strains were likely to become resistant at the antibiotic concentration levels used.

The effectiveness of *B. longum* NCC 2705 was not surprising and may be due to the fact that 70% of all isolated *B. longum* strains normally harbour plasmids (Sgorbati *et al.*, 1982). In addition, *B. longum* strains were already successfully transformed by five research groups (Missich *et al.*, 1994; Argnani *et al.*, 1996; Matsumura *et al.*, 1997; Rossi *et al.*, 1997; F. Arrigoni, personal communication).

In some experiments colonies grew on selective agar plates after electroporation which did not harbour any plasmids. The hybridisation experiments with these colonies proved furthermore that the plasmid had not integrated into the genome. Therefore, these colonies were regarded as spontaneous mutants. Nevertheless, with some of these colonies a PCR fragment, which corresponded to the size of the resistance marker gene, could be amplified using either the primer pair cstet/cstet2.rev or cscat2/cscat2.rev, respectively. This phenomenon can only be explained by a false positive PCR result.

In consequence, it was assumed that the antibiotic concentration in the selective agar plates was too low or the plates were too old and allowed therefore growth of spontaneous mutants after several days.

4.2.6 Transformation efficiency in dependence of DNA amount and origin

To transform bifidobacteria, the pCSC1 plasmid DNA was isolated from *E. coli* XL1-Blue, since the plasmid constructs were first introduced in *E. coli* to improve DNA yield. The transformation experiments with this plasmid DNA yielded low efficiencies of 3-5 *B. longum* NCC 2705 colonies per µg DNA. Park *et al.* (2000) observed in *B. longum* similar transformation efficiencies of $1.2x10^1$ to $2.6x10^2$ cfu/µg DNA with a vector plasmid deriving from pKJ36. In other studies, bifidobacteria transformants were obtained with an almost 100 to 1000 fold higher frequency of $2x10^2$ to $7x10^4$ transformants/µg DNA (Missich *et al.*, 1994; Argnani *et al.*, 1996; Matsumura *et al.*, 1997). These results could be compared with $1.4x10^2$ transformants/µg DNA reached with the control plasmid pRDC102 in this study. The difference between this thesis and the other reports was the selection marker used.

While all other research groups selected on chloramphenicol, spectinomycin or erythromycin, here tetracycline was applied.

Electrotransformation experiments of other high G+C, Gram-positive bacteria also resulted in low transformation efficiencies of $2x10^0$ to $1.6x10^3$ for propionibacteria (Stierli, 2001) and $2x10^3$ for corynebacteria (Haynes and Britz, 1990).

The number of transformants, carrying plasmid pCSC1, could be 10 to 100 fold increased to $3x10^1$ and $3x10^2$, in using plasmid DNA isolated from bifidobacteria instead of *E. coli*. To test whether bifidobacteria have difficulties in taking up the methylated DNA of *E. coli* XL1-Blue as described for propionibacteria by Stierli (2002), the plasmids were introduced into the *E. coli* JM110 *dam* and *dcm* deficient strain. DNA was isolated and introduced into bifidobacteria, but there was no increased transformation rate detectable.

The reintroduction of reisolated plasmid DNA from *B. longum* NCC 2705 transformants into *E. coli* XL1-Blue showed no decreased transformation rate. This gave evidence for the ability of pCSC1 to serve as *Bifidobacterium-E. coli* shuttle vector.

The group of Rossi *et al.* (1998) found that some plasmids could only transform *B. longum, B. animalis* or *B. bifidum* when the transformed DNA had been isolated from *B. infantis* or *B. magnum* instead of *E. coli.* They suggested that some plasmids had to be properly modified prior to transformation. There was no evidence which plasmid constructs had to be modified first by bifidobacteria prior to transformation and which plasmids could be introduced into bifidobacteria strains directly. In general, DNA introduced into bacteria by electroporation is more vulnerable to restriction nucleases than the one transferred by conjugation or natural transformation systems. In using homologous DNA for electroporation the restriction barriers are eluded and transformation is more efficient.

As a further experiment, the influence of the DNA amount for transformation efficiency was examined. The results showed an almost linear increase of efficiency from 0.4µg DNA to a maximum with 0.8µg per assay and then a decreasing efficiency with higher DNA amounts. The experiment demonstrated the importance of DNA concentration for transformation efficiency by using electroporation.

For application in industrial fermentation processes, it is essential that the vector is stably maintained in the host cell, possibly even without selective pressure. The segregational stability of the vector pCSC1 was analysed by growing plasmid harbouring *B. longum* NCC 2705 under selective and non-selective growth conditions. For over 20 generations pCSC1 was stably maintained under selective conditions without decreased growth rate of the host strain.

But under non-selective conditions 60% of the host strains lost pCSC1 after 8 generations and after 14 generations the number of transformants carrying pCSC1 decreased to less than 10^2 colony forming units per ml.

The segregational instability of the 6.9-kb plasmid pCSC1 might be connected with its size. This claim is supported by the report of Rossi *et al*. (1997). The segregational stability tests of Rossi in *B*. *animalis* with the small plasmid pTRE3 (2.8kb) resulted in stability over 100 generations without selective pressure but with plasmid pDG7 (7.2kb) a plasmid loss of 93% was observed. They concluded that segregational stability must be negatively correlated with plasmid size.

4.2.7 Expression of *tet*(W) or *gfpuv* in *B. longum* NCC 2705 encoded on pCSC1 or pCSC8, respectively

B. *longum* NCC 2705 transformants, carrying plasmid pCSC1, were tested for their minimal inhibitory concentration (MIC) of tetracycline. The 100 fold increased tetracycline MIC (see table 3-6) gave evidence that *B*. *longum* NCC 2705 could express *tet*(W) under the control of a pAP1 promoter.

B. *longum* NCC 2705 was also transformed with the *gfpuv*-reporter vector pCSC8. Plasmid DNA could only be reisolated from a sole transformant at a low amount (see figure 3-17, lane 4), suggesting that the plasmid could not replicate well. This was obviously caused by the introduction of the *gfpuv* gene into pCSC1, since pCSC1 is able to replicate stably in transformants, though under selective pressure. Additionally, the transformant lost plasmid pCSC8 after 2 fresh inoculations of overnight cultures, even under selective pressure. Conceivably, the replication and stability problems of pCSC8 were caused by its size of 7.8kb. As already mentioned the plasmid size may be negatively correlated with the segregational stability. Therefore, the plasmid might be too large for the rolling circle replication mechanism of pAP1.

However, no expression of the GFPuv protein under the control of a pAP1 promoter was detectable. Only the group of Rossi *et al.* (1998) reported transformation experiments with heterologous genes such as a cholesterol oxidase operon from *Streptomyces* sp., an α-amylase gene from *Bacillus licheniformis* and a lipase gene from *Pseudomonas fluorescens*. They also found that none of these genes was expressed in bifidobacteria.

Transformation experiments with plasmid pCSC9, carrying the *gfpuv* gene in opposite reading direction, produced no transformants at all.

In these experiments the *gfpuv* gene proved to be useless as reporter of bifidobacteria transformants. To perpetuate the expression of *gfpuv* in bifidobacteria, the gene must be controlled by a strong bifidobacteria promoter and must be inserted into another, smaller shuttle vector.

4.2.8 Transformation experiments of *Corynebacterium glutamicum* ATCC 13032 with plasmids pCSC6 and pCSC7

Corynebacteria as well as bifidobacteria belong to the Gram-positive and high G+C branch of bacteria. The attempt was made to transform *C. glutamicum* ATCC 13032 with pCSC6 and pCSC7 (see chapter 3.6). Transformed corynebacteria were detected on selective agar plates containing 5µg/ml chloramphenicol. These results clearly demonstrated that a *Bifidobacterium* plasmids could replicate in *C. glutamicum* and that the chloramphenicol-acetyltransferase gene could be expressed probably under the control of its own promoter.

This experiment showed the functionality of the *cat* gene of the constructed plasmids, yet these vectors could not transform bifidobacteria in this study.

In addition, the attempt was made to transform *B. animalis* DSMZ 20105 and *B. infantis* DSMZ 20088^T with the *Corynebacterium-E. coli* shuttle vector pEC-K18mob2 containing a kanamycin resistance marker gene (see chapter 3.5.3), yet no transformants were obtained on selective agar plates containing 25µg/ml kanamycin. In contrast to these results, Argnani *et al.* (1996) transformed the same *B. animalis* strain with two different *Corynebacterium* vectors also containing a kanamycin resistance gene. With this experiment Argnani demonstrated the functionality of a *Corynebacterium* origin of replication in *Bifidobacterium*. The difference between the Argnani study and this study was that Argnani selected transformants by the chloramphenicol-acetyltransferase gene. This indicated that the kanamycin resistance gene used in pEC-K18mob2 did not seem to work in bifidobacteria.

4.3 Concluding remarks and outlook

The aim of this work was to engineer a *Bifidobacterium-E. coli* shuttle vector. For lack of suitable plasmids of human faecal bifidobacteria, pLME201 and pLME202 were chosen as origins. These two plasmids were based on the *B. asteroides* plasmid pAP1 and were formerly constructed in our laboratory (Kaufmann, 1998).

Two different selection markers were introduced into these plasmids, the chloramphenicol-acetyltransferase (*cat*) gene from *Staphylococcus aureus* plasmid pC194 and the *tet*(W) resistance gene from *Bifidobacterium* sp.

Thus, 7 different possible vectors named pCSC1 to pCSC7 were obtained. Only pCSC1, containing the *tet*(W) gene, could successfully transform *B. longum* NCC 2705 as a sole *Bifidobacterium* strain with a transformation efficiency of $3x10^2$ transformants/μg DNA. pCSC6 and pCSC7, containing the *cat* gene, could transform *Corynebacterium glutamicum* ATCC13032 but none of the tested *Bifidobacterium* strains. All other transformation experiments with other vectors or other bifidobacterial strains failed.

The successful transformations followed the modified methods of Argnani *et al.* (1996) or the method described by Rossi *et al.* (2000b).

The vector plasmid pCSC1 might not be ideal for industrial use and for further genetic engineering of bifidobacteria, because of the loss of pCSC1 after 14 generations under non-selective conditions.

In this study another series of experiments had been carried out with the reporter vectors pCSC8 and pCSC9. These vectors are successors of pCSC1, carrying the commercially available *gfpuv* gene. One *B. longum* NCC 2705 transformant was obtained with pCSC8, but only very low DNA amounts could be reisolated. The transformant lost the plasmid after two fresh inoculations, even under selective pressure. Furthermore, no expression of the green fluorescence protein could be detected.

To prosecute the results of this thesis, more effective transformation techniques, especially for bifidobacteria species, have to be developed.

Further studies are essential with regard to the expression ability of the constructed vectors. Within these studies the interference between replication mechanisms of the vectors and the *gfpuv* gene as well as the functionality of the antibiotic resistance gene promoters should be analysed. Perhaps the insertion of a strong bifidobacterial promoter into the vectors would be useful to increase transformation efficiency and expression levels.

5 References

Akkermans, A. D. L., E. G. Zoetendal and W. M. de Vos (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64**: 3854-3859.

Anderson, D. G. and L. L. McKay (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**: 549-552.

Argnani, A., R. J. Leer, N. van Luijk and P. H. Pouwels (1996). A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. Microbiology **142**: 109-114.

Arrigoni, E., F. Jörger, B. Kollöffel, I. Roulet, M. Herensperger, L. Meile and R. Amadò (2002). *In vitro* fermentability of a commercial wheat germ preparation and its impact on the growth of bifidobacteria. Food Res. Int. **35**: 475-481.

Aspiras, M. B., K. M. Kazmerzak, P. E. Kolenbrander, R. McNab, N. Hardegen and H. F. Jenkinson (2000). Expression of green fluorescent protein in *Streptococcus gordonii* DL1 and its use as a species-specific marker in coadhesion with *Streptococcus oralis* 34 in saliva-conditioned biofilms *in vitro*. Appl. Environ. Microbiol. **66**: 4074-4083.

Atlas, R. M. (1999). Probiotics - snake oil for the new millennium? Environ. Microbiol. **1**: 377-382.

Ballongue, J. (1993). Bifidobacteria and probiotic action. In: Lactic acid bacteria. S. Salminen and A. von Wright (eds.). New York, Marcel Dekker Inc.**:** 357-428.

Barbosa, T. M., K. P. Scott and H. J. Flint (1999). Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurence of *tet*(O) in ruminal bacteria. Environ. Microbiol. **1**: 53-64.

Beerens, H. (1990). An elective and selective isolation medium for *Bifidobacterium* spp. Lett. Appl. Microbiol. **11**: 155-157.

Berg, R. D. (1998). Probiotics, prebiotics or 'conbiotics'? Trends in Microbiol. **6**: 89-92.

Biavati, B. and P. Mattarelli (1991). *Bifidobacterium ruminantium* sp. nov. and *Bifidobacterium merycicum* sp. nov. from the rumens of cattle. Int. J. Syst. Bacteriol. **41**: 163-168.

Biavati, B., P. Mattarelli and F. Crociani (1991). *Bifidobacterium saeculare*: a new species isolated from feces of rabbit. System. Appl. Microbiol. **14**: 389-392.

Biavati, B., B. Sgorbati and V. Scardovi (1992). The Genus *Bifidobacterium*. In: The prokaryotes. 2nd ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K. H. Schleifer (eds.). New York, Springer-Verlag**:** 816-833.

Bourget, N., J. M. Simonet and B. Decaris (1993). Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and rrn loci number. FEMS Microbiol. Lett. **110**: 11-20.

Breed, R. S., E. G. D. Murray and N. R. Smith, Eds. (1957). Bergey's manual of determinative bacteriology.7th ed. Baltimore, Williams and Wilkins.

Brigidi, P., B. Vitali, E. Swennen, L. Altomare, M. Rossi and D. Matteuzzi (2000). Specific detection of *Bifidobacterium* strains in a pharmaceutical probiotic product and in human feces by polymerase chain reaction. System. Appl. Microbiol. **23**: 391-399.

Brosius, J., M. L. Palmer, P. J. Kennedy and H. F. Noller (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Nat. Acad. Sci. USA **75**: 4801-4805.

Buchanan, R. R. and N. E. Gibbons, Eds. (1974). Bergey's manual of determinative bacteriology.8th ed. Baltimore, Williams and Wilkinson.

Charteris, W. P., P. M. Kelly, L. Morelli and J. K. Collins (1997). Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. **35**: 1-27.

Crameri, A., E. A. Whitehorn, E. Tate and W. P. Stemmer (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnol. **14**: 315-319.

Crociani, F., B. Biavati, A. Alessandrini, C. Chiarini and V. Scardovi (1996). *Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries. Int. J. Syst. Bacteriol. **46**: 564-571.

De Vries, W. and A. H. Stouthamer (1967). Pathway of glucose fermentation in relation to the taxonomy of bifidobacteria. J. Bacteriol. **93**: 574-576.

Deguchi, Y., T. Morishita and M. Mutai (1985). Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agric. Biol. Chem. **49**: 13-19.

Dehnert, J. (1957). Untersuchungen über die gram positive Stuhlflora des Brustmilchkindes. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. I Abt. Orig. **169**: 66-83.

Dong, X., Y. Xin, W. Jian, X. Liu and D. Ling (2000). *Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester. Int. J. Syst. Evol. Microbiol. **50**: 119-125.

Feinberg, A. P. and B. Vogelstein (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**: 6-13.

Ferrari, F. A., A. Nguyen, D. Lang and J. A. Hoch (1983). Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154**: 1513-1515.

Feucht, A. and P. J. Lewis (2001). Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264**: 289-297.

Frère, J. (1994). Simple method for extracting plasmid DNA from lactic acid bacteria. Lett. Appl. Microbiol. **18**: 227-229.

Fretz, C. (2000). Beiträge zur Entwicklung eines Transformationssystems für Bifidobakterien. Diplomarbeit im Labor für Lebensmittelmikrobiologie der ETH Zürich. Betreuung: C. Schürch.

Fuller, R. (1989). Probiotics in man and animals. J. Appl. Bacteriol. **66**: 365-378.

Goldenberger, D. (1997). Detection of bacterial pathogens in clinical specimens by broad-range PCR amplification and direct sequencing of part of the 16S rRNA gene. Thesis no 12219. ETH Zurich.

Gómez Zavaglia, A., P. de Urraza and G. DeAntoni (2000). Characterization of *Bifidobacterium* strains using box primers. Anaerobe **6**: 169-177.

Goze, A. and S. D. Ehrlich (1980). Replication of plasmids from *Staphylococcus aureus* in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**: 7333-7337.

Greisen, K., M. Loeffelholz, A. Purohit and D. Leong (1994). PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol. **32**: 335-351.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**: 557-580.

Hartemink, R., B. J. Kok, G. H. Weenk and F. M. Rombouts (1996). Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. J. Microbiol. Meth. **27**: 33-43.

Haynes, J. A. and M. L. Britz (1990). The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. J. Gen. Microbiol. **136**: 255-263.

Heinig, M. J. and K. G. Dewey (1996). Health advantages of breast feeding for infants: A critical review. Nutr. Res. Rev. **9**: 89-110.

Holmes, D. S. and M. Quigley (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193-197.

Hoppler, M. (2001). Transformationsversuche mit dem Plasmid pLME222 in *Actinobacteria*. Diplomarbeit im Labor für Lebensmittelmikrobiologie der ETH Zürich. Betreuung: C. Schürch.

Horinouchi, S. and B. Weisblum (1982). Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150**: 815-25.

Itaya, M., S. M. Shaheduzzaman, K. Matsui, A. Omori and T. Tsuji (2001). Green marker for colonies of *Bacillus subtilis*. Biosci. Biotechnol. Biochem. **65**: 579-583.

Iwata, M. and T. Morishita (1989). The presence of plasmids in *Bifidobacterium breve*. Lett. Appl. Microbiol. **9**: 165-168.

Jerusalem C., K. Soerensen and E. Johansen (2001). Bifidobacterium strain ISO3519 *tetW* gene. unpublished. GenEMBL Accession number AF202986.

Jian, W., L. Zhu and X. Dong (2001). New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. Int. J. Syst. Evol. Microbiol. **51**: 1633-1638.

Kaufmann, P. (1998). Applied genetics and molecular identification of bifidobacteria. Thesis no 12526. ETH Zurich.

Kaufmann, P., A. Pfefferkorn, M. Teuber and L. Meile (1997a). Complete nucleotide sequence of the 2.1kb plasmid pAP1 isolated from *Bifidobacterium asteroides*. Direct submission to database: Accession number Y11549.

Kaufmann, P., A. Pfefferkorn, M. Teuber and L. Meile (1997b). Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63**: 1268-1273.

Kleessen, B., L. Hartmann and M. Blaut (2001). Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora. Brit. J. Nutr. **86**: 291-300.

Kleessen, B., B. Sykura, H. J. Zunft and M. Blaut (1997). Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. Am. J. Clin. Nutr. **65**: 1397-1402.

Kruse, H. P., B. Kleessen and M. Blaut (1999). Effects of inulin on faecal bifidobacteria in human subjects. Brit. J. Nutr. **82**: 375-382.

Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson and G. W. Welling (1995). Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61**: 3069-3075.

Lauer, E. (1990). *Bifidobacterium gallicum* sp. nov. isolated from human feces. Int. J. Syst. Bacteriol. **40**: 100-102.

Lebet, V., E. Arrigoni and R. Amadò (1998). Measurement of fermentation products and substrate disappearance during incubation of dietary fibre sources with human faecal flora. Lebensm. -Wiss. u. -Technol. **31**: 473-479.

Lim, K. S., D. S. Huh and Y. J. Baek (1993). Antimicrobial susceptibility of bifidobacteria. J. Dairy Sci. **76**: 2168-2174.

Mangin, I., Y. Bouhnik, N. Bisetti and B. Decaris (1999). Molecular monitoring of human intestinal *Bifidobacterium* strain diversity. Res. Microbiol. **150**: 343-350.

Martineau, B. (1999). Comparison of four media for the selection of bifidobacteria in dog fecal samples. Anaerobe **5**: 123-127.

Matsuki, T., K. Watanabe, R. Tanaka and H. Oyaizu (1998). Rapid identification of human intestinal bifidobacteria by 16S rRNA- targeted species- and group-specific primers. FEMS Microbiol. Lett. **167**: 113-121.

Matsumura, H., A. Takeuchi and Y. Kano (1997). Construction of *Escherichia coli-Bifidobacterium longum* shuttle vector transforming *B. longum* 105-A and 108-A. Biosci. Biotech. Biochem. **61**: 1211-1212.

Mattarelli, P., B. Biavati, A. Alessandrini, F. Crociani and V. Scardovi (1994). Characterization of the plasmid pVS809 from *Bifidobacterium globosum*. New Microbiol. **17**: 327-331.

Matteuzzi, D., P. Brigidi, M. Rossi and D. Di (1990). Characterization and molecular cloning of *Bifidobacterium longum* cryptic plasmid pMB1. Lett. Appl. Microbiol. **11**: 220-223.

Meile, L. (1998). Mikroorganismen in Lebensmitteln: Umsetzung des probiotischen Konzepts. Lebensmittel-Technologie **31**: 68-72.

Meile, L., W. Ludwig, U. Rueger, C. Gut, P. Kaufmann, G. Dasen, S. Wenger and M. Teuber (1997). *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. System. Appl. Microbiol. **20**: 57-64.

Meile, L., L. M. Rohr, T. A. Geissmann, M. Herensperger and M. Teuber (2001). Characterization of the D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase gene (*xfp*) from *Bifidobacterium lactis*. J. Bacteriol. **183**: 2929-36.

Metchnikoff, E. (1907). The prolongation of life: optimistic studies. London, Heinemann.

Missich, R., B. Sgorbati and D. J. LeBlanc (1994). Transformation of *Bifidobacterium longum* with pRM2, a constructed *Escherichia coli-B. longum* shuttle vector. Plasmid **32**: 208-211.

Mitsuoka, T. (1990). Bifidobacteria and their role in human health. J. Ind. Microbiol. **6**: 263-267.

Miyake, T., K. Watanabe, T. Watanabe and H. Oyaizu (1998). Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. Microbiol. Immunol. **42**: 661-667.

Nebra, Y. and A. R. Blanch (1999). A new selective medium for *Bifidobacterium* spp. Appl. Environ. Microbiol. **65**: 5173-5176.

Olsen, G. J., C. R. Woese, and R. Overbeek (1994). The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176**: 1-6.

O'Riordan, K. and G. F. Fitzgerald (1999). Molecular characterisation of a 5.75-kb cryptic plasmid from *Bifidobacterium breve* NCFB 2258 and determination of mode of replication. FEMS Microbiol. Lett. **174**: 285-294.

Orla-Jensen, M. (1924). La classification des bactéries lactiques. Le lait **4**: 468-474.

Park, M. S., K. H. Lee and G. E. Ji (1997). Isolation and characterization of two plasmids from *Bifidobacterium longum*. Lett. Appl. Microbiol. **25**: 5-7.

Park, M. S., D. W. Shin, K. H. Lee and G. E. Ji (2000). Characterization of plasmid pKJ36 from *Bifidobacterium longum* and construction of an *E.coli-Bifidobacterium* shuttle vector. Microbiol. Biotechnol. **10**: 312-320.

Rada, V. and J. Koc (2000). The use of mupirocin for selective enumeration of bifidobacteria in fermented milk products. Milchwissenschaft **55**: 65-67.

Reuter, G. (1963). Vergleichende Untersuchung über die Bifidus-flora im Säuglings- und Erwachsenenstuhl. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. I Abt. Orig. **191**: 486-501.

Rossi, M., L. Altomare, A. Gonzalez Vara y Rodriguez, P. Brigidi and D. Matteuzzi (2000a). Nucleotide sequence, expression and transcriptional analysis of the *Bifidobacterium longum* MB 219 *lacZ* gene. Arch. Microbiol. **174**: 74-80.

Rossi, M., P. Brigidi and D. Matteuzzi (1997). An efficient transformation system for *Bifidobacterium* spp. Lett. Appl. Microbiol. **24**: 33-36.

Rossi, M., P. Brigidi and D. Matteuzzi (1998). Improved cloning vectors for *Bifidobacterium* spp. Lett. Appl. Microbiol. **26**: 101-104.

Rossi, M., P. Brigidi and D. Matteuzzi (2000b). Electroporation of *Bifidobacteria*. In: Electrotransformation of Bacteria. N. Eynard and J. Teissie (eds.). Berlin, Springer-Verlag**:** 72-77.

Roy, D. and S. Sirois (2000). Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of the *ldh* gene. FEMS Microbiol. Lett. **191**: 17-24.

Roy, D., P. Ward and G. Champagne (1996). Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int. J. Food Microbiol. **29**: 11-29.

Rycroft, C. E., M. R. Jones, G. R. Gibson and R. A. Rastall (2001). A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol. **91**: 878-887.

Salminen, S., E. Isolauri and T. Onnela (1995). Gut flora in normal and disordered states. Chemotherapy **41**: 5-15.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: a laboratory manual. New York, Cold Spring Harbor Laboratory Press.

Satokari, R. M., E. E. Vaughan, A. D. Akkermans, M. Saarela and W. M. de Vos (2001). Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67**: 504-513.

Scardovi, V. (1986). Genus *Bifidobacterium* Orla-Jensen. In: Bergery's Manual of Systematic Bacteriology, 2nd ed. P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (eds.). Baltimore, Williams & Wilkins**:** 1418-1434.

Scardovi, V. and L. D. Trovatelli (1974). *Bifidobacterium animalis* (Mitsouka) comb. nov. and the "*minimum*" and "*subtile*" groups of new Bifidobacteria found in sewage. Int. J. Syst. Bacteriol. **24**: 21-28.

Scott, K. P., C. M. Melville, T. M. Barbosa and H. J. Flint (2000a). Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrob. Agents Ch. **44**: 775-777.

Scott, K. P., D. K. Mercer, A. J. Richardson, C. M. Melville, L. A. Glover and H. J. Flint (2000b). Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. FEMS Microbiol. Lett. **182**: 23-27.

Sgorbati, B., V. Scardovi and D. Leblanc (1982). Plasmids in the genus *Bifidobacterium*. J. Gen. Microbiol. **128**: 2121-2131.

Sgorbati, B., V. Scardovi and D. J. Leblanc (1986a). Related structures in the plasmid profiles of *Bifidobacterium asteroides, B. indicum* and *B. globosum*. Microbiologica **9**: 443-454.

Sgorbati, B., V. Scardovi and D. J. Leblanc (1986b). Related structures in the plasmid profiles of *Bifidobacterium longum*. Microbiologica **9**: 415-422.

Smeianov, V. V., B. A. Efimov, V. M. Korschunov and J. L. Steele (2002). Characterization of plasmid p4M from *Bifidobacterium pseudocatenulatum*. unpublished. GenEMBL Accession number AF359574.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503-517.

Stackebrandt, E., F. A. Rainey and N. L. Ward-Rainey (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. Int. J. Syst. Bacteriol. **47**: 479-491.

Stierli, M. P. (2002). DNA transformation of propionibacteria based on plasmids pLME106 and pLME108. Thesis no 14468. ETH Zurich.

Tanaka, R. and M. Mutai (1980). Improved medium for selective isolation and enumeration of *Bifidobacterium*. Appl. Environ. Microbiol. **40**: 866-869.

Tannock, G. W. (1999). Probiotics: A critical review. Wymondham, Horizon Scientific Press.

Tannock, G. W., A. L. McCartney and W. Wenzhl (1996). Molecular analysis of the composition of the bifidobacterial and *Lactobacillus* microflora of humans. Appl. Environ. Microbiol. **62**: 4608-4613.

Terzaghi, B. E. and W. E. Sandine (1975). Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**: 807-813.

Teuber, M. (2000). Fermented milk products. In: The microbiological safety and quality of food. B. M. Lund, T. C. Baird-Parker and G. W. Gould (eds.). Gaithersburg, Maryland, Aspen. **1:** 535-589.

Tissier, H. (1900). Recherches sur la flore intestinales des nourrissons (Etat normal et pathologique). Thèse de médecine de l'Université de Paris.

Vandamme, P., B. Pot, P. De Vos, K. Kersters and J. Swings (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60**: 407-438.

Ventura, M., R. Reniero and R. Zink (2001). Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl. Environ. Microbiol. **67**: 2760-2765.

Wahl, G. M., M. Stern and G. R. Stark (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**: 3683-3687.

Watabe, J., Y. Benno and T. Mitsouka (1983). *Bifidobacterium gallinarum* sp. nov.: a new species isolated from the ceca of chickens. Int. J. Syst. Bacteriol. **33**: 127-132.

Webb, C. D. and O. Resnekov (1999). Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization in *Bacillus subtilis*. In: Green fluorescent protein. M. Conn (eds.). San Diego, Academic Press. **302:** 136-153.

Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**: 697-703.

Wells, J. M., P. W. Wilson and R. W. F. Le Page (1993). Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J. Appl. Bacteriol. **74**: 629-636.

Yanisch-Perron, C., J. Vieira and J. Messing (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**: 103-119.

Yazid, A. M., A. M. Ali, M. Shuhaimi, V. Kalaivaani, M. Y. Rokiah and A. Reezal (2000). Antimicrobial susceptibility of bifidobacteria. Lett. Appl. Microbiol. **31**: 57-62.

Curriculum vitae

Born January 22, 1973, in Huttwil, Switzerland

1979-1989	Primary education in Reiden and Zofingen
1989-1993	Secondary education in Zofingen (Kantonsschule Zofingen)
1993	Matura Type C
1993-1998	Study of Food Engineering at the Swiss Federal Institute of Technology in Zurich, Switzerland (Dipl. Lm.-Ing. ETH)
1999-2002	Scientific collaborator and assistant at the Laboratory of Food Microbiology at the Swiss Federal Institute of Technology in Zurich, Switzerland, PhD Thesis