Doctoral Thesis

Nucleotide sequences as a basis to genetic and taxonomic investigations of Acetobacter species isolated from industrial fermentations

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Publication Date:
1998

Permanent Link:
https://doi.org/10.3929/ethz-a-001945787

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Nucleotide Sequences as a Basis to Genetic and Taxonomic Investigations of *Acetobacter* Species isolated from Industrial Fermentations

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

(ETHZ)

for the degree of

Doctor of Technical Sciences

presented by

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born January 15, 1968
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accepted on the recommendation of

Prof. Dr. M. Teuber, examiner
Prof. Dr. Th. Leisinger, co-examiner
I would like to thank Prof. Michael Teuber for his supervision and interest in this thesis and Dr. Martin Sievers for his support and helpful ideas.

I thank Prof. Thomas Leisinger for his kind acceptance to co-examine this thesis.

Thanks to all co-workers in the Laboratory of Food Microbiology for all their help and for having contributed to a certainly most memorable 4 years in my life.

The support of the Swiss Federal Institute of Technology is gratefully acknowledged.
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Summary

Acetic acid bacteria are divided into the two genera *Acetobacter* and *Gluconobacter*. These Gram-negative, strictly aerobic bacteria have a strong oxidizing capacity for a variety of sugars, sugar acids and alcohols. The strains used in vinegar production are further characterized by a high resistance to acetic acid. The main biochemical differentiation characteristics of the isolates from high percentage vinegar productions against the traditional species are both a strong tolerance to acetic acid and the requirement of acetic acid for growth. Based on phenotypic and genotypic data, *Acetobacter europaeus* has been described as a main species of industrial vinegar fermenters in central Europe (Sievers et al., 1992). Differentiation of *Acetobacter*-strains from industrial vinegar fermentations remains difficult due to their unwillingness to grow well on semisolid media. Data of DNA similarities (30 to 100%) among industrial strains has revealed their division into more than one group suggesting the occurrence of several new species (Kneubühler, 1994; Sievers and Teuber, 1995).

In this thesis two experimentally accessible molecules, the small plasmid pAEU601 of the *Acetobacter europaeus* type strain and the 23S rRNAs of the *A. europaeus* and the *A. xylinus* type strains and a strain isolated from a Kombucha beverage were isolated and sequenced.

The 3.8 kbp plasmid pAEU601 was chosen as a model molecule to analyse the gene structure of a plasmid from high acidic *Acetobacter* production strains. Cloning of the complete linearized plasmid in *E. coli* and *E. faecalis* was not possible. The two clones obtained were pRJ1, the large *Hind*II fragment of pAEU601 ligated to pUC18, and pRJ3, the PCR amplified fragment of pAEU601, which contains the small *Hind*II fragment, ligated to the pGEM®-T vector. Both clones possess the ori region for replication in *E. coli* and the gene coding for resistance to Ampicillin of pUC plasmids.

The complete sequence of plasmid pAEU601 provides a basis for more detailed molecular biology of *Acetobacter europaeus* and related strains. Analysis of the open reading frames of pAEU601 revealed homologies to mobilization genes and virulence associated regions of other plasmids, no homology of an open reading frame on pAEU601 with the sequence of a known replication gene was found. There is also no apparent homology of pAEU601 with plasmids previously described in *Acetobacter* and *Gluconobacter* strains.
The usefulness of the 23S rDNA sequence as a source of target sites for oligonucleotide probes was investigated. Such probes could be used for rapid differentiation between Acetobacter strains. Comparison and alignment of the 23S rrna genes of the three closely related strains Acetobacter europaeus, a high acetic acid production strain, Acetobacter intermedius from a Kombucha beverage and Acetobacter xylinus confirmed their close genetic relatedness and revealed useful sites for the design of oligonucleotide probes. The oligonucleotide probe 23seu which is based on the 23S rRNA of the A. europaeus type strain was not A. europaeus species-specific, but turned out to be useful for the differentiation of strains that belong to a new species in the genus Acetobacter, Acetobacter intermedius. None of the strains belonging to the species A. intermedius hybridized with the oligonucleotide probe 23seu. The species status of A. intermedius is based on 16S rDNA sequence alignments, missing hybridization signals with the oligonucleotide probe 23seu, DNA-DNA hybridization data and phenotypic properties.
Zusammenfassung


In dieser Arbeit wurden zwei leicht zu isolierende Moleküle, das kleine Plasmid pAEU601 aus dem *A. europaeus*-Typstamm und die 23S rRNAs der *A. europaeus* und *A. xylinus*-Typstämme und eines aus einem Kombucha-Getränk isolierten Stammes sequenziert.

Über die Nucleotidsequenz des 3.8 kB grossen Plasmides pAEU601 soll der Einstieg in die Plasmidgenetik der Hochleistungs-Essigbakterien erreicht werden. Das Klonieren des kompletten, linearisierten Vektors in *E. coli* und in *E. faecalis* war nicht möglich. In verschiedenen Versuchen wurden schliesslich die zwei Klone pRJ1 und pRJ3 erhalten. pRJ1 ist das grosse HindIII-Fragment von pAEU601 ligiert mit pUC18, und pRJ3 ist ein mittels PCR amplifizierter Teil von pAEU601, der das kleine HindIII-Fragment enthält, ligiert mit dem Vektor pGEM®-T. Beide rekombinante Plasmide enthalten eine ori-region für die Replikation in *E. coli* und das Gen für die Ampicillin-Resistenz der pUC-Vektoren.

Die vollständige Sequenz vom Plasmid pAEU601 bildet die Basis für eine detailliertere molekulare Forschung von *Acetobacter europaeus* und verwandten Stämmen. Die Analyse der offenen Leseraster auf pAEU601 hat gezeigt, dass Homologien derselben mit Mobilisations-Genen und virulenzassozierten Regionen auf anderen Plasmiden bestehen. Es wurde hingegen keine Homolgie mit bekannten Replikationsregionen oder -genen auf Plasmiden gefunden,
auch besteht keine Ähnlichkeit mit den Plasmid-Sequenzen anderer Acetobacter- und Gluconobacter-Stämmen.


Der Speziesstatus von A. intermedius basiert auf 16S rDNA Sequenzvergleichen, fehlenden Hybridisationssignalen mit der Sonde 23seu, DNA-DNA Homologiewerten unter 70% mit A. europaeus und phänotypischen Merkmalen. Im Gegensatz zu A. europaeus-Stämmen, welche ohne Essigsäure im Medium nicht oder sehr schlecht wachsen, und A. xylinus-Stämmen, welche Essigsäurekonzentrationen von 3% im Medium nicht tolerieren, ist A. intermedius befähigt, sowohl mit als auch ohne Essigsäure im Medium zu wachsen.
1 Introduction

1.1 Acetic Acid Bacteria

Ethanol- or sugar-containing plant sap is the natural habitat of acetic acid bacteria which are divided into the two genera *Acetobacter* and *Gluconobacter*. The Gram-negative, strictly aerobic, rod shaped bacteria are characterized by a strong oxidizing capacity for a variety of sugars, sugar acids and alcohols, and a high resistance to acetic acid. A number of these bioconversions are exploited on an industrial scale, e.g., in the production of acetic acid for vinegar, gluconic acid as a cleansing product and acidifier in foods, ketogluconates, hydroxyacetone as a suntanning agent, L-sorbose from D-sorbitol in the process of vitamin C production, cellulose formation, and production of the Kombucha beverage by an association of acetic acid bacteria with yeasts (Swings, 1992; Sievers *et al*., 1995).

The main use of industrial strains in vinegar production is to produce a high concentration of acetic acid. Submerged vinegar fermentations are initiated by inoculation with ‘seed vinegar’, a microbiologically undefined fermentation broth from previous fermentations. The starting concentration of new mash is 9.7% (w/v) acetic acid and 4.5% ethanol. The lack of defined pure starter cultures is due to problems in isolation and the difficulty of conservation of *Acetobacter* strains responsible for high acid (10-14% acetic acid) production (Sievers and Teuber, 1995). Isolation and cultivation of these strains was achieved by the streaking of first fermentation broth and then the pure culture onto agar plates containing 4% acetic acid and 3% ethanol and incubation at a relative humidity of 95% (Entani *et al*., 1985; Sievers *et al*., 1992). The basic and industrial interest in the resistance to acetic acid (survival in an extremely acidic environment with a pH of 2.5) and the dependance on acetic acid for growth by industrial *Acetobacter* strains has led to studies of these characterisms on a gene (Fukaya *et al*., 1990; Fukaya *et al*., 1993) and on a protein level (Lasko *et al*., 1997; Sievers *et al*., 1997). Thurner (1997) has recently described the genetic and proteinacious basis of ethanol metabolism in *Acetobacter europaeus*, a main component of submerged vinegar production in Europe (Sievers *et al*., 1992), and the taxonomic relationship of this strain to the metabolically closely related strain *Acetobacter polyoxogenes* from Japan (Entani *et al*., 1985).
1.2 Plasmids in *Acetobacter europaeus*

The presence of plasmids in all the investigated *Acetobacter* strains has allowed the use of plasmid profiling to compare the microflora of different fermenters in different vinegar plants and to monitor the stability, origin and identity of a culture (Teuber et al., 1987; Sievers, 1989; Kneubühler, 1994). The number of plasmids and their size is thought to be strain specific and in 1994 Kneubühler described the *A. europaeus* type strain as showing 9 plasmid bands upon electrophoresis (Kneubühler, 1994). However, during the last four years only 3 plasmids (in the sizes of 3.8kbp, ca. 16kbp and ca. 27kbp) were detected in the *A. europaeus* type strain. This could be due to either differences in plasmid isolation (persons and/or material) or to the fact that the plasmid profile is not a stable trait in this strain (possible causes of instability could be IS-elements, phage infections or spontaneous plasmid curing).

Successful construction of vectors and their transfer and stable maintenance in acetic acid bacteria has been described previously for *A. aceti* (Fukaya et al., 1985), *A. pasteurianus* (Grones et al., 1993) and *A. xylinus* (Valla et al., 1986). Dobrowolski and Gründig, 1990, describe transposon mutagenesis of *A. xylinus*. So far, only one transformation experiment with a high acetic acid producing strain has been performed (Tayama et al., 1994). Other transformation experiments of acetic acid bacteria with plasmid DNA were performed with strains with low tolerances to acetic acid such as *A. aceti* (Okumura et al., 1985), *A. xylinus* (Hall et al., 1992) and *G. oxydans* (Creaven et al., 1994).

Cloning experiments with the smallest (3.8 kbp) of the three endogenous plasmids present in the *A. europaeus* type strain have been performed previously (Vela, 1995; Cruceli, 1995) without success. So far, no readily identifiable phenotypic properties (e.g. antibiotic or metal ion resistance marker) associated with the naturally occurring *A. europaeus* plasmids are known. The plasmid appears in high copy number and in more than one *A. europaeus* strain. This plasmid was named pAEU601 for plasmid *Acetobacter europaeus* DSM6160 Nr 1 and more closely analyzed to see if it would be a useful cloning vector.
1.3 Taxonomy of Acetic Acid Bacteria

The taxonomic relationships of these unique microorganisms was investigated by DNA-DNA hybridization, ribosomal RNA operon sequencing, development of genus- and species-specific gene probes and electrophoretic protein patterns. DNA/rRNA hybridization studies have shown that Acetobacter and Gluconobacter species are closely related and are placed in the family Acetobacteraceae (Gillis and De Ley, 1980). The division into two genera is based on physiological and genetical features. Acetobacter species are clearly distinguished from Gluconobacter species by their ability to further oxidize acetate to CO$_2$ and H$_2$O (De Ley, 1970). Molecular phylogeny based on small subunit rRNA sequences from Acetobacter and Gluconobacter species were determined to construct phylogenetic trees reflecting their distant and close relationships. Together with Rhodopila, Acidiphilium and Acidocella they represent a cluster of acidophilic bacteria with a distinct line of descent in the $\alpha$-subclass of Proteobacteria (Sievers et al., 1994 and 1995). The metabolism of acetic acid bacteria and their phylogenetic positioning firmly establishes them in a group of extremophilic bacteria.

1.3.1 Acetobacter europaeus

Differentiation of Acetobacter-strains from industrial vinegar fermentations is difficult due to their unwillingness to grow well on semisolid media. Data of DNA-DNA homologies (30 to 100%) among industrial strains revealed their division into more than one group suggesting the occurrence of several new species. Based on these data Acetobacter europaeus was described as the main species of industrial vinegar fermenters in central Europe (Sievers et al., 1992). The main biochemical differentiation characteristics of the A. europaeus isolates against the traditional species are both a strong tolerance to acetic acid, 4 to 8% on solid media and 10-14% in industrial fermentations, and a requirement of acetic acid for growth.

The most closely related species to A. europaeus is A. xylinus with a 99.6% 16S rDNA sequence similarity (Sievers et al., 1994). The species status of representatives of these two species is supported by DNA-DNA hybridization data (the DNA homology between A. europaeus and A. xylinus is <30%) and phenotypic differences (Sievers et al., 1992). The high homologies of the 16S rRNA primary structure did not allow for the construction of an oligonucleotide probe to differentiate between A. europaeus and A. xylinus. One aim of this thesis was the sequencing of the 23S rRNA gene in the hope of finding more appropriate target sites for oligonucleotide probes.
1.3.2 *Acetobacter intermedius*, sp. nov.

Phenotypic and genotypic investigations of strains from industrial vinegar fermentations (cider- and spirit vinegar) and from a tea fungus beverage (Kombucha) made a group of *Acetobacter*-strains stand out which grew in media with 3% acetic acid and 3% ethanol as does *A. europaeus*, and did, however, not require acetic acid for growth. One Kombucha isolate (TF2) was chosen, its 16S rDNA and 23S rDNA were sequenced and DNA-DNA hybridizations with other *Acetobacter* and *Gluconobacter* strains were performed.

Previously Kneubühler, 1994, characterized total cellular proteins of 44 *Acetobacter* and *Gluconobacter* strains SDS-PAGE (Kersters, 1985) to provide a reproducible phenotypic property for these strains. Computerized numerical analysis of the protein profiles showed that the 25 different strains from industrial vinegar fermentations clustered in 4 phenons separated from all other investigated strains with a close linkage of *A. xylinus* (Euzeby, 1997) to the *A europaeus* group. The protein patterns correlated with the results of DNA-DNA hybridizations with the *A. europaeus* type strain (Sievers and Teuber, 1995).

The low (30-<70%) DNA-DNA hybridization similarity group (Sievers et al., 1992) and the 4 phenons described by protein profiling (Kneubühler, 1994; Sievers and Teuber, 1995) were analyzed on a phenotypic and genotypic level and compared to an isolate (TF2) from a tea fungus beverage (Sievers et al., 1995). Based on 16S rDNA sequence alignments, missing hybridization signals with an *A. europaeus*-specific oligonucleotide probe, DNA-DNA hybridization data and phenotypic properties, strain TF2 is described as the new species *Acetobacter intermedius* in the genus *Acetobacter*.

1.4 Topics of this thesis

This thesis describes the isolation and sequencing of 2 experimentally accessible molecules: a small plasmid of the *Acetobacter europaeus* type strain and the 23S rRNAs of *A. europaeus*, *A. xylinus* and an *Acetobacter* strain isolated from a Kombucha beverage. The first complete sequence of a plasmid from a high acidic production strain should give information about its replication modus and its possible use as a vector. The large *rrn* subunit was sequenced to elucidate the taxonomy of the three obviously related but not identical microorganisms.
2 Materials and Methods

2.1 Bacterial strains and plasmids

Acetic acid bacteria used in this study are shown in Table 1. Other bacterial strains and plasmids are shown in Table 2.

Table 1. Acetic acid bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain designation</th>
<th>source</th>
<th>original substrate / fermenter type&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Acetobacter aceti</td>
<td>NCIB 8621&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSM 3508</td>
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<td>Acetobacter pasteurianus</td>
<td>LMD 22.1&lt;sup&gt;T&lt;/sup&gt;</td>
<td>LMG 1262</td>
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<td>Acetobacter liquefaciens</td>
<td>IFO 12388&lt;sup&gt;T&lt;/sup&gt;</td>
<td>LMG 1328</td>
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<tr>
<td>Acetobacter hansenii</td>
<td>NCIB 8746&lt;sup&gt;T&lt;/sup&gt;</td>
<td>LMG 1527</td>
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<td>Acetobacter xylinus</td>
<td>NCIB 11664&lt;sup&gt;T&lt;/sup&gt;</td>
<td>LMG 1515</td>
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<tr>
<td>Acetobacter xylinus</td>
<td>NCIB 613</td>
<td>LMG 1510</td>
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<tr>
<td>Acetobacter xylinus</td>
<td>De Ley 25</td>
<td>LMG 25</td>
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<tr>
<td>Acetobacter xylinus</td>
<td>ATCC 23768</td>
<td>DSM 2004</td>
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<td>Acetobacter xylinus</td>
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<td>DSM 46604</td>
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<td>Acetobacter methanolicus</td>
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<td>Gluconobacter oxydans</td>
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<td>Acetobacter europaeus</td>
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<td>Acetobacter europaeus</td>
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<td>LM ETH</td>
<td>generator</td>
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<tr>
<td>Acetobacter europaeus</td>
<td>WW</td>
<td>LM ETH</td>
<td>submersed</td>
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<tr>
<td>Acetobacter europaeus</td>
<td>S1</td>
<td>LM ETH</td>
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<td>ZIM B021</td>
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<td>Acetobacter europaeus</td>
<td>V1</td>
<td>ZIM B026</td>
<td>wine vinegar / submersed</td>
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<tr>
<td>Acetobacter sp.</td>
<td>TSN5</td>
<td>LM ETH</td>
<td>generator</td>
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</table>
Acetobacter sp. TSN1 LMETH generator
Acetobacter sp. TSA8 LMETH generator
Acetobacter intermedius TF2\textsuperscript{T} DSM 11804 Kombucha
Acetobacter intermedius JK3 ZIM B022 cider vinegar / submersed
Acetobacter intermedius JKD ZIM B025 cider vinegar / submersed
Acetobacter intermedius TSA10 LMETH generator
Acetobacter intermedius OSSPR LMETH submersed
Acetobacter intermedius TSN3 LMETH generator
Acetobacter intermedius TSA3 LMETH generator
Acetobacter intermedius TSA7 LMETH generator
Acetobacter intermedius KRI LMETH submersed
Acetobacter intermedius TSN4 LMETH generator
Acetobacter intermedius E1 LMETH submersed
Acetobacter intermedius E2 LMETH submersed
Acetobacter intermedius OSSW LMETH submersed
Acetobacter sp. HE LMETH submersed

\textsuperscript{T} = type strains

ZIM = Zbirka Industrijskih Mikroorganizmov, Ljubljana, Slovenia
LM ETH = Labor für Lebensmittelmiobiologie, ETH Zurich, Switzerland
DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen
LMG = Laboratorium voor Microbiologie, Rijksuniversiteit, Gent

\textsuperscript{a)} submersed = submersed fermentation
generator = trickle fermenter

Table 2. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain and/or plasmid</th>
<th>Relevant properties</th>
<th>Application</th>
<th>Reference</th>
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<tr>
<td>\textit{Escherichia coli}</td>
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<tr>
<td>DH5\textalpha{}</td>
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<tr>
<td>LacZ\textsuperscript{-}</td>
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<td>Allows blue/white color selection for lacZ containing plasmids</td>
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<tr>
<td>Hanahan, 1983</td>
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<td>HB101</td>
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<td>RecA\textsuperscript{-}</td>
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<td>Negative control for DNA hybridization experiments</td>
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<tr>
<td>Boyer \textit{et al.}, 1969</td>
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### Materials and Methods

<table>
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<tr>
<th>XL-1 Blue</th>
<th>Tet', LacZ'</th>
<th>Allows blue/white color selection for pUC plasmids</th>
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#### Enterococcus faecalis

<table>
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<tr>
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<th>Plasmid-free, Rif' Fus'</th>
<th>Recipient for conjugation and transformation</th>
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#### Lactococcus lactis subsp. cremoris

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<th>AC1</th>
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<th>Neve et al, 1984</th>
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#### Plasmids

<table>
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<th>Description</th>
<th>Source</th>
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<td>pACYC184</td>
<td>Te' Cm'; 4.2 kb</td>
<td>Chang et al., 1978</td>
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<td>pBR322</td>
<td>Ap' Tet'; 4.36 kb</td>
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<td>pCL1921</td>
<td>Spec' Str'; 4.6 kb</td>
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<tr>
<td>pGEM®-T</td>
<td>Ap' LacZ'; 3.0 kb</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>pNZ18</td>
<td>Km' Cm'; 5.7 kb</td>
<td>W. de Vos, NIZO, Holland</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap' LacZ'; 2.68 kb</td>
<td>Yanish-Perron et al., 1985</td>
</tr>
<tr>
<td>pAEU601</td>
<td>cryptic; 3.8 kb</td>
<td>from A. europaeus DSM 6160 this work</td>
</tr>
<tr>
<td>pRJ1</td>
<td>Ap' LacZ'; 4.5 kb</td>
<td>small HindII (1.96 kb) fragment from pAEU601 in pUC18 this work</td>
</tr>
<tr>
<td>pRJ3</td>
<td>Ap' LacZ'; 5.0 kb</td>
<td>PCR-fragment (2.06 kb) of pAEU601 in pGEM®-T this work</td>
</tr>
</tbody>
</table>
2.2 Media and growth conditions

2.2.1 Acetobacteraceae

Acetobacter europaeus and Acetobacter intermedius strains were cultivated on modified AE-medium containing 0.5% glucose, 0.3% yeast extract, 0.4% peptone, 0.9% agarose, 3%(v/v) ethanol and 3%(v/v) acetic acid. Incubation on solid media was at 30°C and 92-96% relative air humidity (Sievers et al., 1992). AEG20% is AE-medium with 20% glucose. Incubation in liquid media was performed in baffled Erlenmeyer flasks on a rotatory shaker. Acetobacter and Gluconobacter type strains were cultivated in GY-medium containing 5% glucose and 1% yeast extract; a concentrated glucose-solution was autoclaved separately to avoid Maillard reactions. Incubation was at 30°C. GYG20% is GY-medium containing 20% glucose. YP-medium is medium containing 0.3% yeast extract, 0.4% peptone and 0.9% agarose. To analyze growth behavior with different sugars, alcohols and acids, YP-medium containing 0.5% acetic acid (YPA) was supplemented with 20% of the according carbon source. YPA20% is YPA medium containing 20% acetic acid.

2.2.2 Escherichia coli

Escherichia coli strains HB101 and XL-1 Blue were cultivated on Luria-Bertani-medium at 37°C (Sambrook et al., 1989). Antibiotics were used in the following concentrations for the selection of plasmids and vectors: tetracycline 10μg/ml; ampicillin 50μg/ml. Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were added to obtain final levels in LB agar plates of 0.04mM and 40μg/ml, respectively, for blue or white colony selection of recombinant pUC18 and pGEM®-T transformants of E. coli XL-1 Blue.

2.3 Formation of 2- and 5-ketogluconic acid

The formation of 2- and 5-ketogluconic acid was assayed with Acetobacter intermedius strain TF2 cultivated in shaken culture in 2% glucose, 0.5% yeast extract, 0.3% peptone and 2.0% acetic acid for 14 to 21 days. 2- and 5-ketogluconic acid were determined by thin layer chromatography of growth medium (Goessele et al., 1980).
2.4 Methods of DNA isolation and gel electrophoresis

2.4.1 Preparation of chromosomal DNA from *Acetobacter* strains

Chromosomal DNA was prepared by a modification of the method of Marmur (1961). *Acetobacter europaeus* was grown as in 2.2.1. For a typical preparation a cell sediment of up to 1g wet weight was used. The (frozen) sediment was washed twice with a 50mM Tris, 1mM EDTA - buffer (pH 8.0) and then resuspended in 1ml of freshly prepared lysozyme solution (2.5mg lysozyme/ml, 25mM Tris pH 8.0, 50mM glucose, 10mM EDTA) and incubated for 20 min. at 37°C. 20µl proteinase K solution (0.2% (w/v) proteinase K in 50mM Tris (pH 8.0), preincubated for 30 min. at 37°C) and 50µl 10% (w/v) SDS-solution were added and incubated for 1h. The solution was then extracted twice with phenol and methylenechloride-isoamylalcohol (24:1, v/v). The upper aqueous phase was then removed into another cap and precipitated with 0.1 volume of 3M sodium-acetate (pH 5.2) and 1 volume of isopropanol. Incubation on ice for 1h was followed by centrifugation for 30 min. at 4°C. The resulting pellet was washed with 70% (v/v) ethanol, dried (Speed Vac, Savant, USA) and resuspended in 100-300µl bidistilled water.

2.4.2 Large scale plasmid isolation from *Acetobacter europaeus*

If not otherwise stated, solutions had a temperature of 4°C and procedures were performed at 4°C.

250ml of broth containing cells in their exponential growth phase were harvested by centrifugation at 4'000g for 10 min. The cell pellet (1-2g) was suspended in 20ml of wash buffer (50mM Tris, 1mM EDTA, pH 8.0), sedimented again under the same conditions and then suspended in 0.75ml TS-buffer (50mM Tris, 25% sucrose, pH 8.0). Lysis of the cells was started by addition of 0.5ml TS-buffer containing 10mg/ml lysozyme and 5µg/ml RNAse. The RNAse stock solution (5mg enzyme per ml of 50mM sodium acetate buffer, pH 5.0) was heated for 10 min. at 90°C before use.

Incubation of the mixture was for 15 min. at 37°C. In the following step the suspension was mixed with 0.3ml 0.25M EDTA, pH 8.0, and kept on ice for 10 min. After warming to room temperature, 0.3ml 20% (w/v) sodium dodecyl sulfate (SDS) in 30mM Tris, 5mM EDTA, pH 8.0, was added and the suspension mixed on a vortex mixer (Vortex Genie, Bender&Hobein, Switzerland) three times for 1-2 sec. to shear the chromosomal DNA.
Alkaline denaturation of the DNA was by addition of 1N NaOH to get a final pH of 12.3 to 12.5 in the solution. A 5-min. incubation was followed by the addition of 0.4ml of 2M Tris, pH 7.0. The neutralized suspension was very gently mixed and incubated for 5min. Precipitation of chromosomal DNA and proteins was achieved by addition of 0.5ml 5M NaCl and incubation for 1-2 h on ice. The precipitated material was removed by centrifugation at 14'000g for 20 min.
The supernatant was diluted with 2ml distilled water and extracted twice with 3ml phenol saturated with 3% aqueous sodium chloride. Phase separation was obtained by centrifugation for 20 min. at 2'800g and 10°C. The aqueous upper phase was treated with 3ml cold methylene chloride / isoamyl alcohol (24:1, v/v) to remove any remaining protein and phenol, and cleared by centrifugation for 20 min. at 2'800g and 10°C. The aqueous phase was mixed with 1/10 of its volume of 3M aqueous sodium acetate and 2 volumes of 96% ethanol (-20°C). Precipitation of plasmid DNA was over night at -20°C. The precipitated DNA was collected by centrifugation for 20 min. at 2'800g and 4°C. The supernatant was discarded, the sediment dried for 15 min. (Speed Vac, Savant, USA) and resuspended in 100μl 10mM Tris, 1mM EDTA, pH 8.0.

2.4.3 Large scale plasmid isolation from Lactococcus lactis AC1
Isolation of plasmid DNA of Lactococcus lactis AC1 was performed as in chapter 2.4.2.

2.4.4 Large scale plasmid isolation from E. coli
Plasmids were isolated from a 100ml overnight culture supplemented with the relevant antibiotic by using the 'DNA Purification System' (Wizard™ Plus Midipreps, Promega, Madison, USA) according to the manufacturers instructions.

2.4.5 Small scale plasmid isolation from E. coli
A single colony was inoculated in 5ml LB-medium (Sambrook et. al., 1989) supplemented with the relevant antibiotic and incubated for 5 h at 37°C. A pellet obtained by centrifugation (4'000g) of 2ml of the culture was resuspended in 200μl P1 (50mM Tris, 10mM EDTA, pH 8.0, with 100μg/ml RNAse). First 200μl P2 (1% SDS (w/v), 0.2M NaOH) and then 200μl P3 (3M potassium acetate pH 5.5) were added, after addition of each buffer the suspension was gently mixed. The mixture was then centrifuged (13’000g) for 10 min. 50μl 3M sodium
acetate, pH 5.5, and 500 μl isopropanol were added to 500 μl of the supernatant and centrifuged for 10 min. (13'000g). The supernatant was removed and the pellet was washed with 70% (v/v) ethanol, dried (Speed Vac, Savant, USA) and then resuspended in 50 μl bidistilled water.

2.4.6 Agarose gel electrophoresis

Plasmid DNA for plasmid profiling was analyzed in 0.65% agarose gels in 1x TAE buffer (Sambrook et al., 1989). Plasmid DNA from Lactococcus lactis subsp. cremoris AC1 was used as reference size marker for covalently closed circular (ccc)-forms of plasmids (2.4.3). Digested DNA was separated in 1% agarose gels prepared in 1x TAE buffer. 1kb-DNA ladder (Gibco BRL, Life Technologies AG, Switzerland) was used as DNA size marker for linear DNA fragments. Agarose gel electrophoresis was performed in 1x TAE buffer at 100V (Gel Electrophoresis Apparatus GNA 100, Pharmacia). The agarose gels were stained in a 5μg/ml ethidium bromide (Sigma) solution for 10 min. and incubated in a water bath for 20 min. to remove the surplus of ethidium bromide from the gels. The DNA in the agarose gels was visualized using ultraviolet light at a wavelength of 302nm and then photographed (Camera UVB and Mitsubishi Video Copy Processor).

2.4.7 DNA isolation from agarose

The DNA isolation from agarose gels was performed with the QIAEX Gel Extraction Kit according to the manufacturers instructions (Qiagen, Kontron, Switzerland).

2.5 In vitro amplification of the origin of replication of pUC18

The origin of replication (ori) of pUC18 was amplified in vitro. The 5’- and 3’- primers were TCCTCGCTCAGTACTGCT (positions 2658 to 2677 in pUC18) and CTTCAAGAAGTACGATGTAGCAG (positions 2093 to 2114 in pUC18), respectively. PCR was performed in a total volume of 100 μl containing 2.5 U DynaZyme™ DNA polymerase F-501L (FinnZymes, BioConcept, Switzerland), 10 μl 10x DynaZyme™-reaction buffer, 1 μM of each primer and 300 μM of each deoxynucleoside triphosphate. The mixture was subjected to 35 cycles of amplification in a thermal cycler (Perkin Elmer Cetus 480) with the following parameters: 92°C, 2 min.; 60°C, 1 min.; 72°C, 1 min.
2.6 Ligation and transformation

2.6.1 Restriction enzyme analysis

Restriction enzyme analyses were performed as described by the manufacturer (Boehringer, Mannheim, Germany).

2.6.2 Ligation and transformation in *E. coli* XL-1 Blue by electroporation

Insert DNA and vector DNA were digested with the appropriate restriction enzymes, separated by agarose gel electrophoresis, purified with the QIAEX Gel Extraction Kit (Qiagen, Kontron, Switzerland) and resuspended in bidestilled water. Digested insert- and vector-DNA were mixed (ratio 1:10, vector/insert), heated for 5 min. at 50°C, cooled for 3 min. on ice and dried by vacuum-centrifugation (Speed-Vac, Savant, USA). 15µl of 10x reaction buffer (Boehringer, Mannheim, Germany) and 1µl of bacteriophage T4 DNA ligase (1U/µl) (Boehringer, Mannheim, Germany) were added to the dried DNA mixture without mixing the sample. The ligation mixture was incubated overnight at 18°C and then at 4°C for 2h. *E. coli* XL-1 Blue was transformed by electroporation, using a Bio-Rad Gene Pulser™ and a Pulse Controller™ apparatus according to the Gene Pulser Electroprotocols manual (Bio-Rad Laboratories, 1993, Herkules, CA 94547).

2.6.3 Alkaline phosphatase treatment of the vector pUC18

Bidestilled water and the appropriate amount of NEB 4-buffer (Biolabs, Beverly, MA, USA) were added to the restriction digestion mixture to a final volume of 100µl. 1U calf intestinal phosphatase (CIP) (Biolabs, Beverly, MA, USA) was added and then incubated for 1h at 37°C. CIP was removed with the QIAEX Extraction Kit (Qiagen, Kontron, Switzerland) according to the concentrating protocol.

2.6.4 Cloning of PCR-product into pGEM®-T

PCR-products were ligated into the pGEM®-T vector according to the manufacturers instructions (pGEM®-T and pGEM®-T Easy Vector Systems, Promega, USA). The pGEM®-T vector has a 3' thymidine overhang at the insertion site which greatly improves the efficiency of ligation of a PCR product by preventing recircularization of the vector.
2.7 Cloning and sequencing of plasmid pAEU601

pAEU601 was restricted by HindII to obtain two fragments (Figure 10). The large HindII fragment was cloned into pUC18 at its HindII-site and sequenced. The small HindII fragment was amplified by PCR using primers that bound to the large HindII fragment (Table 3) and the product cloned into pGEM®-T.

2.7.1 In vitro amplification of part of pAEU601

The polymerase chain reaction (PCR) was performed in a reaction mixture of 100μl containing about 10ng of template DNA (ccc DNA of pAEU601), 2mM of each of the four dNTPs (Boehringer, Mannheim), 1pmol of each primer (Table 3; primers were synthesized by Microsynth, Balgach, Switzerland), 2U of DynaZyme™ DNA polymerase FS01L (FinnZymes) and 1x DynaZyme™ buffer. The PCR mixture was overlaid with 80μl of mineral oil. The amplifications were performed on a DNA thermal cycler (Perkin Elmer Cetus 480) with the following parameters: 92°C, 1 min.; 50°C, 1 min.; 72°C, 2 min. 30 sec. and 25 cycles (Jacsman, 1997). Cloning of the PCR-amplified part of plasmid pAEU601 was performed using the pGEM®-T vector system (2.6.4).

Table 3. Oligonucleotide primers for amplification of DNA from plasmid pAEU601

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Binding site on pAEU601</th>
<th>1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr.1</td>
<td>5'AAAATCAGTATCGAACCGCC3'</td>
<td>170-150</td>
<td></td>
</tr>
<tr>
<td>Nr.2</td>
<td>5'CTAGCTTTTCTCTGACCATAA3'</td>
<td>1930-1940</td>
<td></td>
</tr>
</tbody>
</table>

1) Numbers correspond to the nucleotides indicated in Figure 9

2.7.2 Sequencing of pAEU601

The plasmid DNA of the large HindII fragment of pAEU601 in pUC18 (pRJ1) and the PCR-amplified part of of pAEU601 (contains the small HindII fragment) in pGEM®-T (pRJ3) were isolated from E. coli XL-1 Blue using Wizard™ Plus Midipreps (Promega, USA) (Table 2). The nucleotide sequences of the cloned DNA inserts were determined using an ALFExpress DNA Sequencer (Pharmacia Biotech AB, Uppsala, Sweden). Except for the forward and reverse primers of the vector, the synthetic oligonucleotide primers (Microsynth, Balgach, Switzerland) were Cy5™-labeled. The sequencing reactions were performed according to the
manufacturers instructions. To determine the nucleotide sequence of the cloned DNA fragments in pUC18 and pGEM®-T, primers listed in Table 4 were used.

Table 4. pUC18 and pGEM®-T primers and synthetic primers used for sequencing pAEU601 clones

<table>
<thead>
<tr>
<th>Sequence (5' to 3')</th>
<th>Designation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTTTCCACTACGAC</td>
<td>-40</td>
<td>M13/pUC/pGEM®-T forward primer</td>
</tr>
<tr>
<td>CAGGAAACAGCTATGAC</td>
<td>rev</td>
<td>M13/pUC/pGEM®-T reverse primer</td>
</tr>
<tr>
<td>GTATATCTGGAGATGTTTAT</td>
<td>paeu1</td>
<td>anneals in pRJ1</td>
</tr>
<tr>
<td>TTTTAATGTTAATGACTT</td>
<td>paeu2</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>ATCGGCCTCGGCCCTGCTT</td>
<td>paeu3</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>ATGCCGGTTCACATGATTT</td>
<td>paeu4</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>ATTTACATGAACGTGTATTA</td>
<td>paeu5</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>AAACACAATCTTTGGAGTC</td>
<td>paeu6</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>TTGGGAATGCTGTTTGA</td>
<td>paeu7</td>
<td>anneals in pRJ3</td>
</tr>
<tr>
<td>CCAAGCGTTCGTTCAGATA</td>
<td>paeu8</td>
<td>anneals in pRJ1</td>
</tr>
<tr>
<td>AGAGGACAGAGGGGAA</td>
<td>paeu9</td>
<td>anneals in pRJ1</td>
</tr>
</tbody>
</table>

2.7.3 Data analysis of the sequence of pAEU601

Computer analyses of the DNA sequence of pAEU601 were performed using the GCG software package of the University of Wisconsin Genetics Computer Group (Madison, Wis., USA). The sequence and respective open reading frames were further compared to the different databases EMBL, SWISS-PROT and GenBank.
2.8 *In vitro* amplification and sequencing of *rrna* genes

The 16S rDNA of the *Acetobacter intermedium* type strain and the 23S rDNA of the *Acetobacter europaeus*, *Acetobacter intermedium* and *Acetobacter xylinus* type strains were amplified by PCR and sequenced.

### 2.8.1 *In vitro* amplification of the 16S *rrna* gene

A loopful of cells from agar were suspended in a 10mM Tris, 10mM EDTA - buffer (pH 8.0). Cell-lysis was induced by addition of 20μg Proteinase K and incubation at 55°C for 30 minutes. Nucleic acids were purified by extraction with 100μl phenol (saturated with 3% NaCl) and subsequently by extraction with 100μl methylene chloride / isoamyl alcohol (24:1[vol./vol.]). 1-3μl of the aqueous phase were directly used for PCR. 16S rDNA was amplified using oligonucleotide primers complementary to highly conserved regions of bacterial rRNA genes (Amman *et al.*, 1990). The 5’- and 3’-terminal primers were GAGTTTGAT(C/T)(C/A)TGGCTCA (positions 9 to 26, according to the *Escherichia coli* numbering system (Brosius *et al.*, 1981)) and CA(G/T)AAAGGAGGTGATCC (positions 1545 to 1529), respectively. PCR was performed in a total volume of 100μl containing 1 U DynaZyme™ DNA polymerase F-501L (FinnZymes), 10μl 10x DynaZyme™-reaction buffer, 1μM of each primer and 300μM of each deoxynucleoside triphosphate. The mixture was amplified in a thermal cycler (Perkin Elmer Cetus 480) as follows: an initial denaturation at 94°C for 5 min. followed by: 92°C, 1 min.; 47°C, 2 min.; 72°C, 2 min. and 35 cycles.

### 2.8.2 *In vitro* amplification of the 23S *rrna* gene

Preparation of DNA for PCR was as mentioned (2.8.1). The 5’- and 3’-terminal primers were TGCGGCTGGATCACCTCC (positions 3039 to 3056 (Brosius *et al.*, 1981)) and CCCGCTTAGATGCTTTCAGC (positions 6262 to 6243 (Brosius *et al.*, 1981)), respectively. PCR was performed in a total volume of 100μl containing 4 U DynaZyme™ EXT DNA polymerase F-505S (FinnZymes), 10μl 10x DynaZyme™ EXT DNA-reaction buffer, 2.0mM MgCl₂, 1μM of each primer and 360μM of each deoxynucleoside triphosphate. The mixture was subjected to 30 cycles of amplification in a thermal cycler (Biometra Personal Cycler™). An initial denaturation at 93°C for 2 min. was followed by: 93°C, 20 sec.; 60°C, 30 sec.; 68°C, 3 min.
2.8.3 Purification of PCR-product

PCR-products were directly purified with the NucleoSpin Extract kit (Macherey-Nagel, Oensingen, Switzerland). Elution from the DNA binding column was with bidistilled water.

2.8.4 Direct Sequencing of amplified 16S rDNA and 23S rDNA

Automated cycle sequencing of 300ng purified 16S rDNA and 23S rDNA (corresponds to 2-10μl of purified DNA from chapter 2.8.3) was performed using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP according to the manufacturer's instructions (Amersham International plc, Buckinghamshire, England). Synthetic oligonucleotide primers (Microsynth, Balgach, Switzerland) were Cy5™-labeled; the primers and their positions according to the *Escherichia coli* numbering system (Brosius et al., 1981) are listed in Table 5 and Table 6. Unless otherwise indicated sequences of primers were selected from published data on bacteria. Cycle sequencing was performed on a Biometra Personal Cycler™ (Biometra, Göttingen, Germany) with the following parameters: 95°C, 30 sec.; 50°C, 30 sec.; 72°C, 60 sec. and 25 cycles (Dasen et al., 1998). Detection of the denatured (85°C, 3 min.) cycle-sequenced samples was performed on an ALFexpress™ DNA sequencer (Pharmacia, Uppsala, Sweden) using a hydrolink long ranger gel (Long Ranger™, FMC BioProducts, Rockland, USA).

Table 5. Primers for 16S rDNA sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Target site</th>
<th>Specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubac338</td>
<td>5'ACTCTACGGGAGGCAGC3'</td>
<td>338-355</td>
<td>Bacteria</td>
<td>Amann et al., 1990</td>
</tr>
<tr>
<td>MS350</td>
<td>5'CTGCTGCTCCCAGTA3'</td>
<td>350-336</td>
<td>Bacteria</td>
<td>Amann et al., 1990</td>
</tr>
<tr>
<td>uni515</td>
<td>5'ACCGCGGCTGCTGGCAC3'</td>
<td>515-498</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>uni775</td>
<td>5'GGMATGATACCCTGGTAGTCC3'</td>
<td>775-796</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>uni1088</td>
<td>5'GTTAAATCCGCGCAGAC3'</td>
<td>1088-1107</td>
<td>Bacteria</td>
<td>Amann et al., 1995</td>
</tr>
<tr>
<td>uni1392</td>
<td>5'GTACACACCGCAGC3'</td>
<td>1392-1408</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>uni1392r</td>
<td>5'TGACGGCCGTGTGTC3'</td>
<td>1392-1376</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
</tbody>
</table>

1) positions according to the *Escherichia coli* numbering system (Brosius et al., 1981)
### Table 6. Primers for 23S rDNA sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Target site&lt;sup&gt;1)&lt;/sup&gt;</th>
<th>Target site&lt;sup&gt;2)&lt;/sup&gt;</th>
<th>Specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-1</td>
<td>5'-ATACGGGGCTATCACCCG&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>3841-3824</td>
<td>303-330</td>
<td>α-Proteobacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b0</td>
<td>5'-CCTTTCCCTCACGGTACT&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>3974-3957</td>
<td>459-441</td>
<td>Proteobacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b1rev</td>
<td>5'-TTTCGGGGAGAACCAGCTAT&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>4302-4321</td>
<td>785-804</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b1</td>
<td>5'-ATAGCTGTTCCTCCCGAAA&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>4321-4302</td>
<td>804-785</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b2rev</td>
<td>5'-GATGGCTGCTTCTAAGCCAAC&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>4579-4559</td>
<td>1061-1041</td>
<td>Bacteria</td>
<td>this study</td>
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<tr>
<td>b2</td>
<td>5'-GTTGGCTTAGAAGCGACCATC&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>4559-4579</td>
<td>1041-1061</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b3</td>
<td>5'-CCCCTAAGGCAGGCCGAAG&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>4848-4868</td>
<td>1332-1352</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b4</td>
<td>5'-AGAGAATAACCAAGGCCCTTG&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>5130-5194</td>
<td>1511-1530</td>
<td>Proteobacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b5</td>
<td>5'-AAGTTCCGACCTGCACGAAT&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>5452-5471</td>
<td>1807-1826</td>
<td>Bacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b6</td>
<td>5'-GTAACGGAGCGCGCGAT&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>5757-5774</td>
<td>2125-2142</td>
<td>α-Proteobacteria</td>
<td>this study</td>
</tr>
<tr>
<td>b7</td>
<td>5'-AGAACGTCGTGAGACAGTTTC&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>6027-6080</td>
<td>2441-2460</td>
<td>α-Proteobacteria</td>
<td>this study</td>
</tr>
</tbody>
</table>

<sup>1)</sup> positions according to the *Escherichia coli* numbering system (Brosius et al., 1981)

<sup>2)</sup> positions according to Figure 14
2.8.5 Data Analysis of 16S rDNA and 23S rDNA

Computer analyses of the DNA sequences were performed using the GCG software package of the University of Wisconsin Genetics Computer Group (Madison, Wis., USA). The 16S rDNA and 23S rDNA sequences of *A. intermedius* have been deposited in the EMBL Nucleotide Sequence Library (Cambridge, UK) under the accession numbers Y14694 and Y14680, respectively.

2.9 DNA hybridization studies

2.9.1 DNA-DNA hybridization with chromosomal DNA

Total DNA of *Acetobacter* and *Gluconobacter* species was prepared as in 2.4.1. DNA probes (0.1-0.2 µg) for hybridizations were internally labeled with 20 µCi [α-32P]dATP (3000 Ci/mmol) using Klenow Polymerase and the hexanucleotide mixture Nr. 1277081 (Boehringer, Mannheim, Germany) according to the instructions of the manufacturer. Pharmacia NAP-10 columns (Pharmacia, Uppsala, Sweden) were used to remove unincorporated 32P-labeled nucleotides.

100-500 ng of *Acetobacter intermedius* DNA were restricted with EcoRV prior to labeling. Equal amounts (1-2 µg) of EcoRV-restricted DNA from various *Acetobacter* and *Gluconobacter* strains were separated by gelelectrophoresis and vacuum-blotted (Vacu-Blot, Biometra, Switzerland) to a Zeta-Probe® GT blotting membrane (Biorad, Switzerland) according to the method of Southern (1975). The membrane was dried at 80°C and 30 min. to fix the DNA to the membrane. Hybridization with labeled *A. intermedius* probe was performed at 65°C (Sievers et al., 1992) in 0.25M Na2HPO4 (pH 7.2) and 7% SDS according to the standard protocol for Zeta-Probe® GT blotting membranes. Washing of the membrane was twice 15 min. in 20mM Na2HPO4 (pH 7.2) and 5% SDS and then twice in 20mM Na2HPO4 (pH 7.2) and 1% SDS, all washing steps were performed at 65°C. The wet membranes were sealed into plastic bags and the labeled bands were visualized on X-ray films (Fuji RX) with intensifying screens for 1 day to a week.

Analysis of the X-ray films was performed with the program WINCAM, Cybertech, Berlin. The hybridization signal obtained by restricted and labeled DNA-probe with the identical DNA on the membrane was defined as the 100% intensity signal, the intensities of the signals obtained with EcoRV restricted DNA of other *Acetobacter* and *Gluconobacter* strains were calculated by the program WINCAM, calibration of the values was through linear regression.
2.9.2 Oligonucleotide labeling

10 pmol of the respective synthetic oligonucleotide primer (Microsynth, Balgach, Switzerland) was 5'-end-labeled with 25 μCi [γ-32P]dATP (6000 Ci/mmol) using T4 Polynucleotide Kinase according to the manufacturer's instructions (Biolabs, Beverly, MA, USA). Pharmacia NICK columns (Pharmacia, Uppsala, Sweden) were used to remove unincorporated 32P-labeled nucleotides. The probes used in colony hybridization experiments are listed in Table 7 and indicated in Figure 14.

Table 7. Sequence of probes used in colony hybridization experiments

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Target organism</th>
<th>Target site1)</th>
<th>T2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligotf2</td>
<td>5’TGCAGAAACAAACCACCTGGC3’</td>
<td>A. intermedius type strain</td>
<td>2272-2249</td>
<td>42°C</td>
</tr>
<tr>
<td>23 eux</td>
<td>5’ATGCGCCAAAAGCCGGAT3’</td>
<td>A. europaeus strains</td>
<td>3455-3437</td>
<td>45°C</td>
</tr>
<tr>
<td>oligoxyl</td>
<td>5’GACTGTTCCTGATATTACGC3’</td>
<td>A. xylinus type strain</td>
<td>2348-2324</td>
<td>54°C</td>
</tr>
</tbody>
</table>

1) positions according to the Escherichia coli rRNA numbering system (Brosius et al., 1981)
2) hybridization temperature

2.9.3 Preparation of dot blot filters

A loopful of freshly grown cells on solid media were suspended in 1 ml of a 0.45% NaCl- and 0.5% Lysozyme-solution and incubated for 15 min. at 37°C. After centrifugation, 0.9 ml of the supernatant were removed and the cells resuspended in the remaining 0.1 ml. Either 10 μl of the suspension were directly pipetted on to a Zeta-Probe® GT blotting membrane (Biorad, Switzerland) or 100 μl of the suspension applied to a Zeta-Probe® GT membrane by vacuum filtration according to the manufacturer’s instructions (MINIFOLD I, SRC 60D, Schleicher&Schuell, Switzerland). The membrane was dried at 80°C and 30 min. to fix the DNA to the membrane. Denaturation of the nucleic acids was performed on 1 M NaCl saturated 3 MM paper (Whatman, Switzerland) for 4 min. Neutralization and washing steps were performed in the same manner with 1 M Tris pH 8.0 and 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), respectively.
2.9.4 Hybridization conditions

Hybridizations were carried out in a hybridization oven (Hybaid). Hybridization conditions were the relevant temperature for the respective oligonucleotide probe (Table 7) and 0.25M Na₂HPO₄ (pH 7.2) and 7% SDS according to the standard protocol for Zeta-Probe® GT blotting membranes. Washing of the membrane was twice 15 min. in 20mM Na₂HPO₄ (pH 7.2) and 5% SDS and then twice in 20mM Na₂HPO₄ (pH 7.2) and 1% SDS, the washing steps were performed at the same temperature as the hybridization. The wet membranes were sealed into plastic bags and the labeled bands were visualized on X-ray films (Fuji RX) with intensifying screens for 1 day to a week.

2.9.5 Probe stripping and rehybridization

Reprobing was desired to confirm the presence of nucleic acids on the membranes. The Zeta-Probe® GT blotting membrane was prevented from drying between hybridizations, drying of the membrane would irreversibly bind the radioactively labeled probe. Stripping of the membrane was at 95°C for 1.5h in 0.1x SSC and 0.5% SDS. The presence of nucleic acids on the membranes was confirmed by hybridization with [α-³²P]dATP-labeled 23S rDNA of A. europaeus at 65°C.
3 Results

Nucleotide sequencing has been used as a basis to advance our knowledge of important microbiological aspects of high acetic acid producing Acetobacter species.

In chapter 3.1 the results concerning the cloning, sequencing and analysis of the putative open reading frames of plasmid pAEU601 of Acetobacter europaeus are presented. In chapter 3.2 the 23S rDNA sequences of the three closely related species A. europaeus, A. intermedius and A. xylinus are compared and the design of oligonucleotide probes is described. Chapter 3.3 contains the description of the new species Acetobacter intermedius.

3.1 Plasmid pAEU601 from A. europaeus

No information on plasmids from high acid Acetobacter strains has been available. The first complete sequence of such a plasmid from the A. europaeus type strain should give a good idea about the genetic information that it codes for.

3.1.1 Hybridization of pAEU601 with the origin of replication of pUC18

Experiments to make a restriction map of pAEU601 and cloning experiments have been done previously (Vela, 1995 and Cruceli, 1995), both were unsuccessful. Hybridization of pAEU601 with the origin of replication of pUC18 was performed to see whether previous cloning experiments had failed due to incompatibility of pAEU601 and the used cloning vectors. In Figure 1 the Southern hybridization analysis of the plasmid DNA of Acetobacter europaeus with the in vitro amplified origin of vegetative replication (oriV) of pUC18 are shown. The plasmid profile of A. europaeus on the ethidium bromide stained agarose gel shows the three visible plasmids and chromosomal DNA. The ccc (covalently closed circular), the linear and the oc (open circular) form of pAEU601 are indicated. A hybridization signal was only obtained with oriV of pUC18 indicating no significant homology of plasmid pAEU601 of A. europaeus and the origin of vegetative replication of pUC18.
Figure 1. Southern hybridization analysis of plasmid DNA of *Acetobacter europaeus* and *in vitro* amplified origin of vegetative replication (*oriV*) of pUC18. (A) 0.8% TAE agarose gel stained with ethidium bromide. (B) Autoradiography of membrane bound DNA that hybridized with a α-32P-labeled 0.5kb *ori* of pUC18 PCR probe. Lanes: 1, AC1, plasmid profile of *Lactococcus lactis* subsp. *cremoris* AC1 used as plasmid DNA size marker (in kilobases (kb)); 2, plasmid profile of the *A. europaeus* typestrain; 3, *in vitro* amplified *oriV* of pUC18 (536bp)

### 3.1.2 Cloning of linearized and restricted pAEU601 into different vectors

1-4μg of the ccc form of plasmid pAEU601 were obtained from 200ml shaken culture of *Acetobacter europaeus*. As 5-10μg DNA/sequencing reaction are required for sequencing, ligation of pAEU601 to high copy number vectors and cloning of the recombinant plasmids into competent *E. coli* and *E. faecalis* cells which allow rapid isolation of their plasmid DNA was performed.

Only *SalI* and * AvaI* linearize pAEU601 (Vela, 1995). Therefore ligation and cloning experiments were conducted with these enzymes. As no results with linearized plasmid
pAEU601 were obtained, further experiments with *Hind*II, which cuts pAEU601 at two sites (Vela, 1995), were conducted. The results are listed in Table 8, empty fields imply that no cloning experiment was performed. The only clone obtained was the clone of the large *Hind*II fragment RJ1 ligated to pUC18 in *E. coli* XL-1 blue, the clone was named pRJ1. In Figure 2 a map of pRJ1 is shown.

<table>
<thead>
<tr>
<th>organism</th>
<th><em>E. coli</em> XL1-Blue</th>
<th><em>E. faecalis JH2-2</em></th>
<th><em>E. coli</em> DH5α / <em>E. coli</em> XL1-Blue</th>
<th><em>E. coli</em> XL1 Blue</th>
<th><em>E. coli</em> XL1 Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAEU60 1 <em>Sal</em>I</td>
<td>no clone</td>
<td>no clone</td>
<td>no clone</td>
<td>no clone</td>
<td>no clone</td>
</tr>
<tr>
<td>pAEU60 1 <em>Ava</em>I</td>
<td>no clone</td>
<td></td>
<td>no clone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAEU60 1 <em>Hind</em>II</td>
<td></td>
<td></td>
<td>clone of large fragment = pRJ1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Map of clone pRJ1. The vector pUC18 is drawn as a thin line, the β-lactamase coding region (Ap) and the ori region are boxed, the lac operon sequences are shown as a white arrow, the cloned DNA of plasmid pAEU601 is shaded. Positions of primers Nr. 1 and Nr. 2 are indicated by short arrows. The cutting positions of restriction enzymes *Hind*II and *Sal*I are shown.
Figure 3 shows Southern hybridization analysis of restricted and circular plasmid DNA of pAEU601 with $\alpha^{-32}$P-labeled pAEU601. No hybridization signal is obtained with the plasmid profile of _Lactococcus lactis_ subsp. _cremoris_ AC1. pAEU601 hybridizes with itself and not with the other plasmids in _Acetobacter europaeus_ (lane 2). Chromosomal DNA was loaded in lane 3, a fine hybridization band appears at the height of the oc form of pAEU601. The fine hybridization band in lane 4 is isolated ccc form of pAEU601. The hybridization signals in lanes 5 and 6 show that _SalI_ cuts pAEU601 once and _HindIII_ twice. The 1.9 kb _HindIII_ fragment was named RJ1.

(A) 0.8% TAE agarose gel stained with ethidium bromide. (B) Autoradiography of membrane bound DNA that hybridized with $\alpha^{-32}$P-labeled pAEU601. Lanes: 1, AC1, plasmid profile of _Lactococcus lactis_ subsp. _cremoris_ AC1 used as plasmid DNA size marker (in kilobases (kb)); 2, plasmid profile of the _A. europaeus_ typestrain; 3, chromosomal DNA of _A. europaeus_; 4, ccc-form of pAEU601; 5, _SalI_ restricted pAEU601; 6, _HindII_ restricted pAEU601; 7, linear kb ladder
3.1.3 Amplification and cloning of the small HindII fragment

Cloning of the small pAEU601 HindII fragment into pUC18 was not possible. Two primers Nr.1 and Nr.2 (Figure 2; Table 3, 2.7.1) from the cloned and sequenced large HindII fragment RJ1 were chosen and the missing part of plasmid pAEU601 amplified (Figure 4), the amplified fragment was named RJ3. The PCR-product was ligated into pGEM®-T and electroporated into E. coli XL1-Blue. 36 white colonies were analyzed, one of them contained the amplified fragment of 2kb; this clone was named pRJ3. The pGEM®-T vector has a 3’ thymidine overhang at the insertion site which greatly improves the efficiency of ligation of a PCR product by preventing recircularization of the vector.

Figure 4. Gel electrophoresis of amplified fragment of plasmid pAEU601: RJ3. Lanes: 1, linear kb-ladder; 2, PCR-amplified fragment of pAEU601
Restriction of the plasmid pRJ3 with *SphI* and *SalI* (these two enzymes cut in the multiple cloning site of the pGEM®-T vector) revealed 4 fragments (Figure 5 (A), lane 3): unrestricted DNA (pGEM®-T plus amplified fragment), pGEM®-T linearized (3kb), a 1.9kb fragment and a 0.2kb fragment (one *SalI* restriction site in the plasmid and one in the multiple cloning site of the pGEM®-T vector). Restriction of plasmid pRJ1 was performed with *EcoRI* and *HindIII*, these enzymes cut in the multiple cloning site of pUC18. As a control a hybridization with [α-32P]dATP labeled pAEU601 was performed (Figure 5(B)). Hybridization signals were only obtained with the complete plasmid pAEU601 and its cloned fragments, this confirms that only plasmid DNA of pAEU601 was cloned.

![Image of gel and autoradiography](image.png)

Figure 5. Restriction analysis of clones pRJ1 and pRJ3. (A) 0.8% TAE agarose gel stained with ethidium bromide. (B) Autoradiography of membrane bound DNA that hybridized with α-32P-labeled pAEU601. Lanes: 1, linear kb ladder; 2, *EcoRI* and *HindIII* restricted plasmid pRJ1; 3, *SphI* and *SalI* restricted pRJ3; 4, ccc-form of pAEU601
Figure 6. Map of clone pRJ3. The vector pGEM®-T is drawn as a thin line, the β-lactamase coding region (Ap) and the ori region are boxed, the lac operon sequences are shown as a white arrow, the cloned DNA of plasmid pAEU601 is shaded. The restriction sites of the enzymes HindII and SalI are shown.
3.1.4 Determination of nucleotide sequence of plasmid pAEU601

The DNA sequences of the large HindII fragment of pAEU601 in pUC18 (pRJ1, Figure 2) and the small HindII fragment of pAEU601 in pGEM®-T (pRJ3, Figure 6) were determined, the sequencing strategies are shown in Figure 7 and Figure 8.

---

### Figure 7

Position of sequenced strands of pRJ1 (large HindII fragment of pAEU601 in pUC18), pRJ1-40 and pRJ1rev were sequenced with primers that bind to the pUC18 vector. Arrows indicate the direction of the sequencing reactions. A consensus sequence is shown, the bar below the consensus indicates the approximate position in base pairs. The approximate HindII and the SalI restriction sites are indicated by arrows.

---

### Figure 8

Position of sequenced strands of pRJ3 (small amplified HindII fragment of pAEU601 in pGEM®-T), pRJ3rev and pRJ3-40 were sequenced with primers that bind to the pGEM®-T vector. Arrows indicate the direction of the sequencing reactions. A consensus sequence is shown, the bar below the consensus indicates the approximate position in base pairs. The approximate HindII and the SalI restriction sites are indicated by arrows.
3.1.5 Analysis of nucleotide sequence of pAEU601

pAEU601 consists of 3818bp (Figure 9) and is 52.2% in GC content. The sequences of both strands were compared to the sequences in the GenEMBL database with the program „fasta“.

The two optimal scores were a 54.3% identity in a 1400bp overlap with the Salmonella low copy number plasmid pSC101 (Bernardi et al., 1984) and a 55.8% identity in a 701bp overlap with plasmid RSF1010 (Scherzinger et al., 1984), respectively. The sequence was examined in both directions for potential protein coding regions, that is, regions possessing a possible ribosomal binding site and starting with an ATG or GTX codon and terminating after 75 or more coding triplets with any of the three stop codons. 7 such translational ORFs were found, 5 on the R strand and 2 on the L strand (Table 9).

Figure 9. Complete nucleotide sequence of plasmid pAEU601 (EMBL accession numbers is Y17109) oriented from the unique SalI site. Start codons are in bold letters accompanied by a short arrow above or below the sequence indicating the direction of the respective ORF. Primers Nr. 1 and Nr. 2 are boxed. Inverted repeats are indicated by thin arrows.
32 Results

GCTGATTCAGTCGGTCGCTCTTTCTCCGTCACTCAGGATTCGTTAATTTTGCTGATTATTTTG
CGACTTACTTTAGTCAGACACCCGAAAAGAAGAAAGGAATCTATCGTTGCTACGCGCTTCTAAAGAAA
2650

TGCTTATCTTTTCTTTTGGCAGATTTTCTCTACTCTGGTGCTTTTTCAGATCAACAAATGCACTGAGTG
GGAGAAAACCTCTCAAGATAGTTAGAGAAACAAACCCTTTAAACCATTTTTGAAATATAGACATTTATC
2810

CCTCTTTTATTTTTAAAAAACCCCACTTTTTTGACATTTTCTTTTTTGAAATATTTATCTCTTAC
2980

AGGGGAGGAGGAAAGGGCAAAGGCTTCGAGACACTCACGTCATCTTGCTGCTGCTGCTGCTGCTG
3110

ACAGACGCTAAGGGGTTAAGGCTTTTTAAGCCCTAGTTTACCTTTTTCCGGCAGGACATCTTTTG
3350

GAGAAGCTAGTCAGGGAATGGGTGAAGCAGGAAATCGGCTATCAGCACGCTTACACATGGGCCATCCATT
3590
<table>
<thead>
<tr>
<th>Serial No. of reading frames</th>
<th>Position of initiation codon sequence</th>
<th>sequence preceding start codon</th>
<th>No. of amino acids</th>
<th>Polypeptide molecular mass</th>
<th>putative gene or functional region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3014R</td>
<td>GTTTGA&lt;sup&gt;CT&lt;/sup&gt;CCA&lt;sup&gt;AG&lt;/sup&gt;ATTGTT</td>
<td>363</td>
<td>42.1kDa</td>
<td>&lt;i&gt;mob&lt;/i&gt;/relaxation</td>
</tr>
<tr>
<td>1'</td>
<td>3563R</td>
<td>TGC&lt;sup&gt;GG&lt;/sup&gt;CA&lt;sup&gt;C&lt;/sup&gt;C&lt;sup&gt;G&lt;/sup&gt;C&lt;sup&gt;CT&lt;/sup&gt;GG&lt;sup&gt;G&lt;/sup&gt;AAAATCATG</td>
<td>180</td>
<td>20.9kDa</td>
<td>&lt;i&gt;mob&lt;/i&gt;</td>
</tr>
<tr>
<td>2</td>
<td>2838L</td>
<td>CATATAA&lt;sup&gt;T&lt;/sup&gt;AGGTTTTT&lt;sup&gt;T&lt;/sup&gt;A&lt;sup&gt;A&lt;/sup&gt;CAATG</td>
<td>154</td>
<td>17.4kDa</td>
<td>&lt;i&gt;mob&lt;/i&gt;/gyrase</td>
</tr>
<tr>
<td>3</td>
<td>1777L</td>
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<td>75</td>
<td>8.5kDa</td>
<td>virulence associated</td>
</tr>
<tr>
<td>4</td>
<td>1996R</td>
<td>AACGACTGGAG&lt;sup&gt;A&lt;/sup&gt;A&lt;sup&gt;G&lt;/sup&gt;AAGAA&lt;sup&gt;A&lt;/sup&gt;ATG</td>
<td>102</td>
<td>11.6kDa</td>
<td>?</td>
</tr>
<tr>
<td>5</td>
<td>826R</td>
<td>TTCCCCT&lt;sup&gt;C&lt;/sup&gt;T&lt;sup&gt;A&lt;/sup&gt;AGA&lt;sup&gt;G&lt;/sup&gt;AGATAG&lt;sup&gt;G&lt;/sup&gt;TT</td>
<td>203</td>
<td>22.5kDa</td>
<td>?</td>
</tr>
<tr>
<td>6</td>
<td>341R</td>
<td>GATCTGG&lt;sup&gt;G&lt;/sup&gt;C&lt;sup&gt;T&lt;/sup&gt;T&lt;sup&gt;C&lt;/sup&gt;GA&lt;sup&gt;G&lt;/sup&gt;AC&lt;sup&gt;C&lt;/sup&gt;TT&lt;sup&gt;C&lt;/sup&gt;CATG</td>
<td>103</td>
<td>11.9kDa</td>
<td>?</td>
</tr>
<tr>
<td>7</td>
<td>305L</td>
<td>AGATCTATGGAG&lt;sup&gt;C&lt;/sup&gt;C&lt;sup&gt;G&lt;/sup&gt;G&lt;sup&gt;G&lt;/sup&gt;GTTCTGTA</td>
<td>240</td>
<td>26.6kDa</td>
<td>virulence related</td>
</tr>
</tbody>
</table>

Table 9. Location of open reading frames in pAEU601 from the left end to the right end in serial order. The first nucleotide of the translational initiation codon (bold letters) specifies the position for the open reading frame. R and L refer to the rightward (top strand) and leftward (bottom strand) orientation of the reading frame. ORF1' is an internal, in frame, coding sequence of the preceding frame ORF1. The Shine-Dalgarno (Shine and Dalgarno, 1974) sequences are underlined. The last three columns show the number of hypothetical amino acids encoded by the respective open reading frame, the polypeptide molecular mass (kilo Daltons), and the putative gene or functional region assignment, respectively.
Figure 10. Circular map of pAEU601 with ORFs 1-7. Black pointed boxes show the ORFs with homologies to \textit{mob} regions. The shaded arrow indicates the in frame, internal coding sequence. Thick white arrows indicate ORFs with homologies to virulence associated proteins. Thin black arrows indicate ORFs with no apparent homology to known proteins. The restriction sites of the enzymes \textit{HindIII} and \textit{SalI} are shown, as well as the positions of the Primers Nr. 1 and Nr. 2 (pointers).
3.1.6 Analysis of amino acid sequence of ORF1

The derived amino acid sequence of open reading frame 1 has a length of 363 amino acids and was compared to the database Swissprot with the program „fasta“. ORF1 has homologies above 20% with proteins from plasmids which can be mobilized, the % identities and % similarities of the amino acid sequence of ORF1 to the respective amino acid sequences of such plasmids are shown in Table 10. With the program „pepstats“ from the GCG package, the size (44.4kDa) and the isoelectric point (10.5) of the putative protein encoded by ORF1 were calculated. The alignment of ORF1 with the relaxation protein of plasmid pSC101 from S. typhimurium is shown in Figure 11, the figure was calculated with the program „gap“ from the GCG package. Two universally conserved amino acid motifs, motif I and motif II, (Ilyina and Koonin, 1992; Pansegrau et al., 1994) which appear in proteins responsible for DNA replication and plasmid mobilization were found in ORF1 by alignment to 6 other relaxation/mobilization proteins (4 of these proteins are listed in Table 10) with the program „eclustalw“. According to Pansegrau et al. (1994) motif I carries a tyrosine residue at position 22 (position 23 in ORF1), which covalently attaches the protein to the 5’ terminus of the cleaved DNA, and motif III contains a histidine residue essential for relaxase activity (the first histidine of the HisHydrHisHydrHydrHydrHydr motif, where Hydr = bulky hydrophobic residue, (Ilyina and Koonin, 1992)) proposed to activate the aromatic hydroxyl group of the mentioned tyrosine by proton abstraction.

The open reading frame ORF1’ in Table 9 is a possible internal, in frame, coding sequence of ORF1 and has 22% identity and 43% similarity with mobilization protein B of plasmid RSF1010 from E. coli (Scholz et al., 1989).

Table 10. % identities and % similarities of amino acid sequence of ORF1

<table>
<thead>
<tr>
<th>Protein</th>
<th>length of protein</th>
<th>accession number</th>
<th>% identity</th>
<th>% similarity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>relaxation protein of plasmid pSC101 from S. typhimurium</td>
<td>371 amino acids</td>
<td>P14492/X01654</td>
<td>41%</td>
<td>59%</td>
<td>Bernardi et al., 1984</td>
</tr>
<tr>
<td>mobilization protein A of plasmid RSF1010 from E. coli</td>
<td>709 amino acids</td>
<td>P07112/M28829/X04830</td>
<td>33%</td>
<td>55%</td>
<td>Scholz et al., 1989, Derbyshire et al., 1987</td>
</tr>
<tr>
<td>mobilization protein B of plasmid RSF1010 from E. coli</td>
<td>141 amino acids</td>
<td>M28829/X04830</td>
<td>23%</td>
<td>44%</td>
<td>Scholz et al., 1989, Derbyshire et al., 1987</td>
</tr>
<tr>
<td>mobilization protein L of plasmid pTF1 from Thiobacillus ferrooxidans</td>
<td>378 amino acids</td>
<td>X52699/P20085</td>
<td>21%</td>
<td>45%</td>
<td>Drolet et al., 1990</td>
</tr>
</tbody>
</table>
Figure 11. Alignment of ORF1 on pAEU601 with the relaxation protein of plasmid pSC101 from *S. typhimurium*, the figure was calculated with the program „gap“ from the GCG package. ORF 1 is in bold letters. Highly conserved amino acid residues of the conserved motifs I and III are shown with a grey background, the different amino acids in different shades of grey. The HisHydrHisHydrHisHydrHis motif is underlined in both sequences (Hydr = bulky hydrophobic residue). The number above the tyrosine residue indicates its position in the amino acid sequence.
3.1.7 Analysis of amino acid sequence of ORF2

The derived amino acid sequence of open reading frame 2 has a length of 154 amino acids and was compared to the database Swissprot with the program "fasta". With the program "pepstats" from the GCG package the size (17.4kDa) and the isoelectric point (10.6) of the putative protein encoded by ORF2 were calculated. Identities of 25% with the N-terminal region of the gyrase subunit B of both E. coli and S. typhimurium and identities of 23% and 20% with mobilization proteins of the two plasmids RSF1010 and pTF1 were found. The % identities and % similarities of the amino acid sequence of ORF2 to the amino acid sequences of interest are shown in Table 11. The alignment of ORF2 with the mobilization protein C of plasmid RSF1010 from E. coli is shown in Figure 12, the figure was calculated with the program "gap" from the GCG package. ORF 3 is in bold letters.

Table 11. % identities and % similarities of amino acid sequence of ORF2. The question mark in the column "Reference" means that no author is stated under the accession number P06982.

<table>
<thead>
<tr>
<th>Protein</th>
<th>length of protein</th>
<th>accession number</th>
<th>% identity</th>
<th>% similarity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DNA gyrase subunit B</td>
<td>803 amino acids</td>
<td>P06982</td>
<td>25%</td>
<td>43%</td>
<td>?</td>
</tr>
<tr>
<td>S. typhimurium DNA gyrase subunit B</td>
<td>803 amino acids</td>
<td>Q60008</td>
<td>25%</td>
<td>43%</td>
<td>Kratz et al., 1996</td>
</tr>
<tr>
<td>mobilization protein C of plasmid RSF1010 from E. coli</td>
<td>96 amino acids</td>
<td>M28829/X04830</td>
<td>23%</td>
<td>47%</td>
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<tr>
<td>mobilization protein S of plasmid pTF1 from Thiobacillus ferrooxidans</td>
<td>98 amino acids</td>
<td>X52699/P20085</td>
<td>20%</td>
<td>42%</td>
<td>Drolet et al., 1990</td>
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Figure 12. Alignment of ORF2 on pAEU601 with the mobilization protein C of plasmid RSF1010 from E. coli, the figure was calculated with the program „gap“ from the GCG package. ORF 2 is in bold letters.
3.1.8 Analysis of amino acid sequence of ORF3

The derived amino acid sequence of open reading frame 3 has a length of 75 amino acids and was compared to the database Swissprot with the program "fasta". With the program "pepstats" from the GCG package the size (8.5kDa) and the isoelectric point (9.2) of the putative protein encoded by ORF3 were calculated. ORF3 has homologies above 30% with virulence associated proteins and with one unknown hypothetical protein. The % identities and % similarities of the amino acid sequence of ORF3 to the respective proteins are shown in Table 12. The alignment of ORF3 with the virulence associated protein A' from the vap region in *Dichelobacter nodosus* is shown in Figure 13, the figure was calculated with the program "gap" from the GCG package.

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<tr>
<th>Protein</th>
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<th>accession number</th>
<th>% identity</th>
<th>% similarity</th>
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<td>Hypothetical 7.6 kD protein (ORF 1)</td>
<td>72 amino acids</td>
<td>P37371</td>
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<td>62%</td>
<td>Phung et al., 1994</td>
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<tr>
<td>virulence associated protein A' from vap region in <em>Dichelobacter nodosus</em></td>
<td>115 amino acids</td>
<td>Q46561</td>
<td>34.8%</td>
<td>57%</td>
<td>Katz et al., 1994</td>
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<tr>
<td>virulence associated protein A from vap region in <em>Dichelobacter nodosus</em></td>
<td>102 amino acids</td>
<td>Q57475</td>
<td>33.3%</td>
<td>53.3%</td>
<td>Katz et al., 1992</td>
</tr>
<tr>
<td><em>E. coli</em> strain K12 hypothetical protein</td>
<td>120 amino acids</td>
<td>P76124</td>
<td>30%</td>
<td>60.6%</td>
<td>Blattner et al., 1997</td>
</tr>
<tr>
<td>virulence associated protein I from vap region in <em>Dichelobacter nodosus</em></td>
<td>108 amino acids</td>
<td>Q46560</td>
<td>25%</td>
<td>53%</td>
<td>Katz et al., 1994</td>
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Figure 13. Alignment of ORF3 on pAEU601 with the virulence associated protein A' from the vap region in *Dichelobacter nodosus* (Katz et al., 1994), the figure was calculated with the program „gap“ from the GCG package. ORF 3 is in bold letters, the C-terminal 15 amino acids of the virulence associated protein A’ are not shown.
3.1.9 Analysis of amino acid sequence of ORFs 4-6

The derived amino acid sequences of open reading frames 4 (102 amino acids), 5 (203 amino acids) and 6 (103 amino acids) were compared to the database Swissprot with the program „fasta“; no homologies above 20% correlating to the whole length of the respective ORFs were observed.

3.1.10 Analysis of amino acid sequence of ORF 7

The derived amino acid sequence of open reading frame 7 has a length of 240 amino acids, according to the program „pepstats“ it encodes a putative 26.6kDa protein with an isoelectric point of 8.2. Comparison to the data in Swissprot with the program „fasta“ revealed 20% identity and 43% similarity over 224 amino acids with ORF240’ (248 aa) from the virulence-associated locus (vrl) of *Dichelobacter nodosus* (Haring *et al.*, 1995).

3.1.11 Conclusion

Restriction analysis of plasmid pAEU601 was not possible as only incomplete restrictions were obtained. Cloning of *SalI* linearized pAEU601 into pUC18 was not possible. *HindIII* cuts pAEU601 twice, the large fragment RJ1 was cloned into pUC18, the small fragment was only clonable by amplification with PCR (RJ3) and ligation into the pGEM®-T vector. This vector has a 3’ thymidine overhang at the insertion site which improves the ligation efficiency. Comparison of the open reading frames of plasmid pAEU601 to the data in the Genebank revealed homologies to mobilization proteins on plasmids and genes found in virulence associated regions on the genome of *Dichelobacter nodosus*. No homologies to known replication proteins were detected. It remains to be established which of the ORFs is the necessary replication gene.
3.2 23S rDNA sequences of A. europaeus\textsuperscript{T}, A. intermedius\textsuperscript{T} and A. xylinus\textsuperscript{T}

Acetic acid strains used in vinegar production are closely related and difficult to isolate and cultivate. The usefulness of the 23S rDNA sequence as a source of target sites for oligonucleotide probes which could be used as a rapid method to differentiate between Acetobacter strains was investigated. Amplification \textit{in vitro} and sequencing of the 23S rRNA genes of A. europaeus, A. xylinus and an Acetobacter strain isolated from a Kombucha beverage (A. intermedius) were performed to elucidate the taxonomy of the three obviously related but not identical microorganisms.

Development of an oligonucleotide probe for A. europaeus-strains on the basis of 16S rDNA sequences was not possible due to the high similarity to A. xylinus (99.5%, see Table 17) with only 7 varying nucleotides distributed over the length of the gene. The increased degree of sequence variability of the 23S rRNA molecule compared to the 16S rRNA primary structure should offer more appropriate target sites for probes to differentiate between genetically similar organisms (Stackebrandt, 1988, and Röchner and Stackebrandt, 1994).

Figure 14. Alignment of the 23S rDNA sequences of A. europaeus\textsuperscript{T}, A. intermedius\textsuperscript{T} and A. xylinus\textsuperscript{T}. (EMBL accession numbers are X89771, Y14680 and X89812, respectively (Boesch \textit{et al.}, 1998)). Bold and italic letters show the positions of the designed oligonucleotide probes which bind to the respective sequence (see chapter 3.2.1).
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<tr>
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<td>TCTGAATACA TAGGGACATG AGGCGAACCC GGGGAACTGA AACATCTAAG</td>
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A. xylinus  | CGAATGCCTA CAAGCAGTCG GAGCCTCTTA TGGGGTGACG GCGTACCTTT
A. europaeus | CGAATGCCTA CAAGCAGTCG GAGCCTCTTA TGGGGTGACG GCGTACCTTT
Consensus   | ********** ********** ********** ********** **********

A. intermedius | TGTATAATGG GTCAGCGAGT TTCTGTTTGC AGCAAGCTTA AGCCGTTAGG
A. xylinus  | TGTATAATGG GTCAGCGAGT TTCTGTTTGC AGCAAGCTTA AGCCGTTAGG
A. europaeus | TGTATAATGG GTCAGCGAGT TTCTGTTTGC AGCAAGCTTA AGCCGTTAGG
Consensus   | ********** ********** ********** ********** **********

A. intermedius | TGTAGGCGCA GCGAAAGCGA GTCTGAATAG GGCGACGAGT TGCTGGCAGA
A. xylinus  | TGTAGGCGCA GCGAAAGCGA GTCTGAATAG GGCGACGAGT TGCTGGCAGA
A. europaeus | TGTAGGCGCA GCGAAAGCGA GTCTGAATAG GGCGACGAGT TGCTGGCAGA
Consensus   | ********** ********** *******_** ********** **********

A. intermedius | AGACCCGAAA CCGAGTGATC TAGCCATGGC CAGGCTGAAG GTGCGGTAAC
A. xylinus  | AGACCGGAAT CCGAGTGATC TAGCCATGGC CAGGCTGAAG GTGCGGTAAC
A. europaeus | AGACCGGAAT CCGAGTGATC TAGCCATGGC CAGGCTGAAG GTGCGGTAAC
Consensus   | ********** ********** ********** ********** **********

A. intermedius | GTGGCTAGGG GTGAAAGGCC AATCAAACTC GGAAATAGCT GGTTCTCCSC
A. xylinus  | GTGGCTAGGG GTGAAAGGCC AATCAAACTC GGAAATAGCT GGTTCTCCSC
A. europaeus | GTGGCTAGGG GTGAAAGGCC AATCAAACTC GGAAATAGCT GGTTCTCCSC
Consensus   | ********** ********** ********** ********** **********

A. intermedius | GAAATCTATT GAGGTAGACC GTCGAGTATT ACCCCGGGGG GTAGAGCACT
A. xylinus  | GAAATCTATT GAGGTAGACC GTCGAGTATT ACCCCGGGGG GTAGAGCACT
A. europaeus | GAAATCTATT GAGGTAGACC GTCGAGTATT ACCCCGGGGG GTAGAGCACT
Consensus   | ********** ********** ********** ********** **********

A. intermedius | GGATGGGCTA GGGGGGCCCA AAGCCTTACC AAACCTAACC AAACTCCGAA
A. xylinus  | GGATGGGCTA GGGGGGCCCA AAGCCTTACC AAACCTAACC AAACTCCGAA
A. europaeus | GGATGGGCTA GGGGGGCCCA AAGCCTTACC AAACCTAACC AAACTCCGAA
Consensus   | ********** ********** ********** ********** **********
A. intermedius  TACCTCGAAG TATGAGCTCG GCAGACAGAC AGTGGGTGCT AAGGTCCATT
A. xylinus     TACCCTGGAAG TATGAGCTCG GCAGACAGAC AGTGGGTGCT AAGGTCCATT
A. europaeus   TACCCGGAAG TATGAGCTCG GCAGACAGAC AGTGGGTGCT AAGGTCCATT
Consensus      ***-****** ********** ********** ********** **********

A. intermedius  GTCCAGAGGG AAACAGCCCA GACCACCACC TAAGGCCCTT AAATCGTGGG
A. xylinus     GTCCAGAGGG AAACAGCCCA GACCACCACC TAAGGCCCTT AAATCGTGGG
A. europaeus   GTCCAGAGGG AAACAGCCCA GACCACCACC TAAGGCCCTT AAATCGTGGG
Consensus      ********** ********** ********** ********** **********

A. intermedius  TAAGTGGGAA AGGAAGCTGG GATTCCAAAA CAACCAGGAG GTTGGCTTAG
A. xylinus     TAAGTGGGAA AGGAAGCTGG GATTCCAAAA CAACCAGGAG GTTGGCTTAG
A. europaeus   TAAGTGGGAA AGGAAGCTGG GATTCCAAAA CAACCAGGAG GTTGGCTTAG
Consensus      ********** ********** ********** ********** **********

A. intermedius  AAGCAGCCAT CCTTTAAAGA AAGCGTAATA GCTCACTGGT CTAATAGAAA
A. xylinus     AAGCAGCCAT CCTTTAAAGA AAGCGTAATA GCTCACTGGT CTAATAGAAA
A. europaeus   AAGCAGCCAT CCTTTAAAGA AAGCGTAATA GCTCACTGGT CTAATAGAAA
Consensus      ********** ********** ********** ********** **********

A. intermedius  CCCTGCAGCCG AAAATGTAAC GGGGCTCAAG CCACGTGCCG AAGCTGTGGG
A. xylinus     CCCTGCAGCCG AAAATGTAAC GGGGCTCAAG CCACGTGCCG AAGCTGTGGG
A. europaeus   CCCTGCAGCCG AAAATGTAAC GGGGCTCAAG CCACGTGCCG AAGCTGTGGG
Consensus      **_******* ********** ********** ********** **********

A. intermedius  TGCATTCTTT GAATGCAGCG TGGGGGAGGT TTCCGTAGGG CTGTGAAGGA
A. xylinus     TGCATTCTAT GAATGCAGCG TGGGGGAGGT TTCCGTAGGG CTGTGAAGGA
A. europaeus   TGCATTCTAT GAATGCAGCG TGGGGGAGGT TTCCGTAGGG CTGTGAAGGA
Consensus      *****-**-* *_******** ***_****** ********** **********

A. intermedius  GACGGGGTGA CCCTCTCTGG AGATATCGGA AGTGCGAATG CTGACATGAG
A. xylinus     GACGGGGTGA CCCTCTCTGG AGATATCGGA AGTGCGAATG CTGACATGAG
A. europaeus   GACGGGGTGA CCCTCTCTGG AGATATCGGA AGTGCGAATG CTGACATGAG
Consensus      ********** ********** ********** ********** **********

A. intermedius  TAGCGACAAA CAGTGCGAGA AACACTGTCG CCGAAAGTCC AAGGGTTCCT
A. xylinus     TAGCGACAAA CAGTGCGAGA AACACTGTCG CCGAAAGTCC AAGGGTTCCT
A. europaeus   TAGCGACAAA CAGTGCGAGA AACACTGTCG CCGAAAGTCC AAGGGTTCCT
Consensus      ********** ********** ********** ********** **********
A. intermedius  GCGCAAGGTT AATCCGCGCA GGGTGAGCCG GCCCCTAAGG CGAGGGCGAA
A. xylinus    GCGCAAGGTT AATCCGCGCA GGGTGAGCCG GCCCCTAAGG CGAGGGCGAA
A. europaeus  GCGCAAGGTT AATCCGCGCA GGGTGAGCCG GCCCCTAAGG CGAGGGCGAA
Consensus     ********** ********** ********** ********** **********

A. intermedius AGCCGTAGTC GATGGAAACC GGGCAAATAT TCCCGGGCCT GCCAGAAGTG
A. xylinus    AGCCGTAGTC GATGGAAACC GGGCAAATAT TCCCGGGCCT GCCAGAAGTG
A. europaeus  AGCCGTAGTC GATGGAAACC GGGCAAATAT TCCCGGGCCT GCCAGAAGTG
Consensus     ********** ********** ********** ********** **********

A. intermedius ACGAATACAA TATGTTGTCG GGTCTTATCG GATTGATCCG GCTTTTGGAG
A. xylinus    ACGAATACAA TATGTTGTCG GGTCTTATCG GATTGATCCG GCTTTTGGCG
A. europaeus  ACGAATGCAA TATGTTGTCG GGTCTTATCG GATTGATCCG GCTTTTGGCG
Consensus     ******-*** *********_ ********** ********_* ********_*

A. intermedius TATTCCAGGA AATAGCTCTG GCATATAGAC CGTACCCGAA ACCGACACAG
A. xylinus    TATTCCAGGA AATAGCTCTG GCATATAGAC CGTACCCGAA ACCGACACAG
A. europaeus  TATTCCAGGA AATAGCTCTG GCATATAGAC CGTACCCGAA ACCGACACAG
Consensus     -********* ********** ********** ********** **********

A. intermedius GTGGACTGGT AGAGAATACC AAGGCGCTTG AGAGAACGAT GCTGAAGGAA
A. xylinus    GTGGACTGGT AGAGAATACC AAGGCGCTTG AGAGAACGAT GCTGAAGGAA
A. europaeus  GTGGACTGGT AGAGAATACC AAGGCGCTTG AGAGAACGAT GCTGAAGGAA
Consensus     ********** ********** ********** ********** **********

A. intermedius CTAGGCAAAT TACTTGCGTA ACTTCGGGAT AAGCAAGACC CGTCAGTGGG
A. xylinus    CTAGGCAAAT TACTTGCGTA ACTTCGGGAT AAGCAAGACC CGTCAGTGGG
A. europaeus  CTAGGCAAAT TACTTGCGTA ACTTCGGGAT AAGCAAGACC CGTCAGTGGG
Consensus     ********** ********** ********** ********** **********

A. intermedius CAACCATCGG CGGGTGGCAC AGACCAGGGG GTAGCGACTG TTTAGTAAAA
A. xylinus    CAACCATCGG CGGGTGGCAC AGACCAGGGG GTAGCGACTG TTTAGTAAAA
A. europaeus  CAACCATCGG CGGGTGGCAC AGACCAGGGG GTAGCGACTG TTTAGTAAAA
Consensus     *******-** ********** ********** ********** **********

A. intermedius ACACAGGGCT GTGCGAAGTC GAGAGACGAC GTATACGGCC TGACGCCTGC
A. xylinus    ACACAGGGCT GTGCGAAGTC GAGAGACGAC GTATACGGCC TGACGCCTGC
A. europaeus  ACACAGGGCT GTGCGAAGTC GAGAGACGAC GTATACGGCC TGACGCCTGC
Consensus     ********** ********** ********** ********** **********
1701 1750
A. intermedius CCGGTGGCCGG AAGGTAAAGA GGAGGTGTGC AAGCACCGAA TTGAAGCCCC
A. xylinus CCGGTGGCCGG AAGGTAAAGA GGAGGTGTGC AAGCACTGAA TTGAAGCCCC
A. europaeus CCGGTGGCCGG AAGGTAAAGA GGAGGTGTGC AAGCACCGAA TTGAAGCCCC
Consensus ********** ********** ********** ******_*** **********

1751 . 1800
A. intermedius GTAAACGGC GCCTGAACCT ATACGGTCC TAAGGTAGCG AAATTCCTTG
A. xylinus GTAAACGGC GCCTGAACCT ATACGGTCC TAAGGTAGCG AAATTCCTTG
A. europaeus GTAAACGGC GCCTGAACCT ATACGGTCC TAAGGTAGCG AAATTCCTTG
Consensus ********** ********** ********** ********** **********

1801 1850
A. intermedius GGTAAACGGC GGCCGTAACT ATAACGGTCC TAAGGTAGCG AAATTCCTTG
A. xylinus GGTAAACGGC GGCCGTAACT ATAACGGTCC TAAGGTAGCG AAATTCCTTG
A. europaeus GGTAAACGGC GGCCGTAACT ATAACGGTCC TAAGGTAGCG AAATTCCTTG
Consensus ********** ********** ********** ********** **********

1851 1900
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A. xylinus TCGGGTAAGT TCCGACCTGC ACGAATGCGG TAACGACTTC CCCGCTGTCT
A. europaeus TCGGGTAAGT TCCGACCTGC ACGAATGCGG TAACGACTTC CCCGCTGTCT
Consensus ********** ********** ********** ********** **********

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A. xylinus CCAGCATCGG CTCAGCGAAA TTGAATTCCC CGTGAAGATG CGGGGTACCC
A. europaeus CCAGCATCGG CTCAGCGAAA TTGAATTCCC CGTGAAGATG CGGGGTACCC
Consensus ********** ********** ********** ********** **********

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A. xylinus ATCAGAGACA TTCTGTGTAG GATAGGTGGG AGGCTTTGAA ACCGGGGCGC
A. europaeus ATCAGAGACA TTCTGTGTAG GATAGGTGGG AGGCTTTGAA ACCGGGGCGC
Consensus ********** ********** ********** ********** **********

2001 2050
A. intermedius CAGTTCCGGT GGAGCCATCC TTGAATACC ACCCTGACTG TTTCTGATGT
A. xylinus CAGTTCCGGT GGAGCCATCC TTGAATACC ACCCTGACTG TTTCTGATGT
A. europaeus CAGTTCCGGT GGAGCCATCC TTGAATACC ACCCTGACTG TTTCTGATGT
Consensus ********** ********** ********** ********** **********

2051 2100
A. intermedius CTAACCGAGG CCTGTAGCC AGGTCCGGGA CCCTGCATGG TGGGCAGTTT
A. xylinus CTAACCGAGG CCTGTAGCC AGGTCCGGGA CCCTGCATGG TGGGCAGTTT
A. europaeus CTAACCGAGG CCTGTAGCC AGGTCCGGGA CCCTGCATGG TGGGCAGTTT
Consensus ********** ********** ********** ********** **********
A. intermedius GACTGGGCG GTCGGCCTCCC AAAGTGTAAC GGAGGCGCGC GATGGTGGGC
A. xylinus GACTGGGCG GTCGGCCTCCC AAAGTGTAAC GGAGGCGCGC GATGGTGGGC
A. europaeus GACTGGGCG GTCGGCCTCCC AAAGTGTAAC GGAGGCGCGC GATGGTGGGC
Consensus ********** ********** ********** ********** **********

A. intermedius TCAGGCCGGT CGGAAACCGG CTGTCGAGTG CAATGGCATA AGCCCGCCTG
A. xylinus TCAGGCCGGT CGGAAACCGG CTGTCGAGTG CAATGGCATA AGCCCGCCTG
A. europaeus TCAGGCCGGT CGGAAACCGG CTGTCGAGTG CAATGGCATA AGCCCGCCTG
Consensus *****_**** *********_ ********** ********** **********

A. intermedius ACTGTGAGAG TGACAGCTCG ATCAGAGACG AAAGTCGGCC ATAGTGATCC
A. xylinus ACTGTGAGAG TGACAGCTCG ATCAGAGACG AAAGTCGGCC ATAGTGATCC
A. europaeus ACTGTGAGAG TGACAGCTCG ATCAGAGACG AAAGTCGGCC ATAGTGATCC
Consensus ********** ********** ********** ********** **********

A. intermedius GGTGGTCCCG CGTGGAAGGG CCATCGCTCA ACGGATAAAA GGTACTCTAG
A. xylinus GGTGGTCCCG CGTGGAAGGG CCATCGCTCA ACGGATAAAA GGTACTCTAG
A. europaeus GGTGGTCCCG CGTGGAAGGG CCATCGCTCA ACGGATAAAA GGTACTCTAG
Consensus ********** ********** ********** ********** **********

A. intermedius GGATAACAGG CTGATCTCCC CCAAGAGTCC ACATCGACGG GGAGGTTTGG
A. xylinus GGATAACAGG CTGATCTCCC CCAAGAGTCC ACATCGACGG GGAGGTTTGG
A. europaeus GGATAACAGG CTGATCTCCC CCAAGAGTCC ACATCGACGG GGAGGTTTGG
Consensus ********** ********** ********** ********** **********

A. intermedius CACCTCGATG TCGGCTCATC ACATCCTGGG GCTGGAGCAG GTCCCAAGGG
A. xylinus CACCTCGATG TCGGCTCATC ACATCCTGGG GCTGGAGCAG GTCCCAAGGG
A. europaeus CACCTCGATG TCGGCTCATC ACATCCTGGG GCTGGAGCAG GTCCCAAGGG
Consensus ********** ********** ********** ********** **********

A. intermedius TTCGGCTGTT CGCCGATTAA AGTGGTACGT GAGCTGGGTT TAGAACGTCG
A. xylinus TTCGGCTGTT CGCCGATTAA AGTGGTACGT GAGCTGGGTT TAGAACGTCG
A. europaeus TTCGGCTGTT CGCCGATTAA AGTGGTACGT GAGCTGGGTT TAGAACGTCG
Consensus ********** ********** ********** ********** **********

A. intermedius TGAGACAGTT CGGCCCTTAA CTGGGCGGTT TAGAACGTCG
A. xylinus TGAGACAGTT CGGCCCTTAA CTGGGCGGTT TAGAACGTCG
A. europaeus TGAGACAGTT CGGCCCTTAA CTGGGCGGTT TAGAACGTCG
Consensus ********** ********** ********** ********** **********
The sequence similarities of the 23S rRNA genes of *A. europaeus*, *A. intermedius* and *A. xylinus* are shown in Table 13. The 23S rRNA homology of *A. europaeus* and *A. xylinus* are 98.9%, this result correlates with the 99.5% 16S rRNA homology (Sievers et al., 1994) of these two bacteria. *A. intermedius* has 23S rRNA homologies of 99.2% with *A. europaeus* and 99% with *A. xylinus*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Ain</th>
<th>Aeu</th>
<th>Axy</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. intermedius</em></td>
<td>99.2</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td><em>A. europaeus</em></td>
<td></td>
<td></td>
<td>98.9</td>
</tr>
<tr>
<td><em>A. xylinus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 13. Overall 23S rRNA sequence similarity values for *Acetobacter intermedius*, *Acetobacter europaeus* and *Acetobacter xylinus*. Abbreviations: Ain, *Acetobacter intermedius*; Aeu, *Acetobacter europaeus*; Axy, *Acetobacter xylinus*
3.2.1 Design of 23S rDNA oligonucleotide probes

Isolation and cultivation of Acetobacter strains from generators and acetators is a difficult and slow procedure. A possibility for rapid identification of such strains would be by hybridization with a species-specific oligonucleotide probe.

The alignment of the 23S rDNA sequences of the type strains of A. europaeus, A. xylinus and A. intermedius revealed useful target sites for oligonucleotide probes (Figure 14). An oligonucleotide probe named 23seu (Table 15) based on the A. europaeus type strain DNA sequence was tested with different strains and species. 23seu hybridized with Acetobacter strains from our culture collection isolated from generators and acetators in Switzerland, Germany, Slovenia and Spain. It gave no hybridization signal with the A. xylinus type strain NCIB 11664 and the A. xylinus strain NCIB 613, however, hybridized with the following strains which are classified as Acetobacter xylinus: De Ley 25, ATCC 23768 and ATCC 10245. No hybridization signals were obtained with with the type strains of A. intermedius, A. xylinus, A. aceti, A. hansenii, A. liquefaciens, A. methanolicus, A. pasteurianus, Gluconobacter oxydans and Escherichia coli HB101 and also no hybridization signals with strains JK3 and JKD from cider vinegar, strain TF2 (A. intermedius, sp. nov) from a tea fungus beverage and all Acetobacter strains in protein profile phenon 3 (Sievers and Teuber, 1995) (Table 16). Acetobacter strains that gave a positive signal in hybridization experiments with the oligonucleotide probe 23seu derived from the A. europaeus type strain are listed in Table 14. The strains from phenon 4 have DNA reassociation values with A. europaeusT below 30% (this study and Sievers and Teuber, 1995). For strains with rRNA homologies above 95%, DNA reassociation values necessary to indicate the species status (Wayne et al., 1987), therefore the strains TSN5, TSN1 and TSA8 are listed as Acetobacter sp. despite their positive hybridization signal with 23seu. Eventhough the oligonucleotide probe 23seu is not specific for A. europaeus strains, it is useful for differentiation of Acetobacter intermedius as no strains belonging to this species give a positive hybridization signal.

The probe oligoxyl derived from the 23S rDNA of the A. xylinus type strain gave no signal with the A. europaeus-and A. intermedius-type strains, no further testing was performed with other A. xylinus strains. The A. intermedius probe is specific only for Acetobacter intermedius DNA.
<table>
<thead>
<tr>
<th>strain</th>
<th>phenon</th>
<th>identity</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. europaeus</em>†</td>
<td>1</td>
<td><em>A. europaeus</em></td>
<td>DSM 6061</td>
</tr>
<tr>
<td><em>A. europaeus</em></td>
<td>1</td>
<td><em>A. europaeus</em></td>
<td>DSM 6161</td>
</tr>
<tr>
<td>S3</td>
<td>1</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>TSA4</td>
<td>1</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>WW</td>
<td>1</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>S1</td>
<td>2</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>TSA9</td>
<td>2</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>KO</td>
<td>2</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>SW3</td>
<td>2</td>
<td><em>A. europaeus</em></td>
<td>LM ETH</td>
</tr>
<tr>
<td>TSN5</td>
<td>4</td>
<td><em>Acetobacter</em>  sp.</td>
<td>LM ETH</td>
</tr>
<tr>
<td>TSN1</td>
<td>4</td>
<td><em>Acetobacter</em>  sp.</td>
<td>LM ETH</td>
</tr>
<tr>
<td>TSA8</td>
<td>4</td>
<td><em>Acetobacter</em>  sp.</td>
<td>LM ETH</td>
</tr>
<tr>
<td>JK2</td>
<td></td>
<td><em>A. europaeus</em></td>
<td>ZIM B021</td>
</tr>
<tr>
<td>V1</td>
<td></td>
<td><em>A. europaeus</em></td>
<td>ZIM B026</td>
</tr>
<tr>
<td>De Ley 25</td>
<td></td>
<td>„<em>A. xylinus</em>“</td>
<td>LMG 25</td>
</tr>
<tr>
<td>ATCC 23768</td>
<td></td>
<td>„<em>A. xylinus</em>“</td>
<td>DSM 46604</td>
</tr>
<tr>
<td>ATCC 10245</td>
<td></td>
<td>„<em>A. xylinus</em>“</td>
<td>DSM 2004</td>
</tr>
</tbody>
</table>

Table 14. *Acetobacter* species that give a positive signal in hybridization experiments with the oligonucleotide probe 23seu derived from the *A. europaeus* type strain. Empty fields in the column titled „phenon“ indicate that these strains were not included in the protein profile experiments performed by Kneubühler (1994).
Table 15. Sequence of probes used in colony hybridization experiments.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Target organism</th>
<th>Target site(^1)</th>
<th>Target site(^2)</th>
<th>Hybridization Temperature</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligotf2</td>
<td>5'TCAGAAAACACCACTGCC3'</td>
<td><em>A. intermedius</em> type strain</td>
<td>2272-2249</td>
<td>279-261</td>
<td>42°C</td>
<td>this study</td>
</tr>
<tr>
<td>23ceu</td>
<td>5'AATGCGCCAAAAGCCGGAT3'</td>
<td><em>A. europaeus</em> strains</td>
<td>3455-3437</td>
<td>350-331</td>
<td>45°C</td>
<td>this study</td>
</tr>
<tr>
<td>oligoxy1</td>
<td>5'GACTGTCCCTGATACCGC3'</td>
<td><em>A. xylinus</em> type strain</td>
<td>2348-2324</td>
<td>1454-1436</td>
<td>54°C</td>
<td>this study</td>
</tr>
</tbody>
</table>

\(^1\) positions according to the *Escherichia coli* numbering system (*Brosius* et al., 1981)

\(^2\) positions according to the alignment in Figure 14
Table 16. Protein profile homologies within the 4 phenons of strains isolated from generators and acetators in Europe and their DNA-DNA homology with *A. intermedius* and *A. europaeus* (Sievers and Teuber, 1995). Stains which do not fit the described values are underlined.

<table>
<thead>
<tr>
<th>Phenon 1</th>
<th>Strains</th>
<th>Protein profile homology</th>
<th>approximate DNA-DNA homology to <em>A. europaeus</em></th>
<th>approximate DNA-DNA homology to <em>A. intermedius</em></th>
<th>Hybridization with Probe for <em>A. europaeus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSM 6161</td>
<td>77.1 ± 4.4%</td>
<td>90-100%</td>
<td>&lt;60%</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DSM6160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSA4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenon 2</td>
<td>WW S1</td>
<td>87.7 ± 4.6%</td>
<td>ca. 75%</td>
<td>60%</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TSA9 KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SW3 SSPR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenon 3</td>
<td>TSN3 0 SSPR</td>
<td>70 ± 5.9%</td>
<td>&lt;70%</td>
<td>70-100%</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>TSN7 TSA7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSN3 TSA3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSN6 KRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSN10 TSA10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSN4 0 SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HE E1 E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenon 4</td>
<td>TSN5</td>
<td>64.5 ± 1.6%</td>
<td>&lt;30%</td>
<td>ca. 80%</td>
<td>yes, except <em>A. xylinus</em> NCIB 11664</td>
</tr>
<tr>
<td></td>
<td>TSN1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSN8 NCIB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11664</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3 Acetobacter intermedius, sp. nov.

Acetobacter europaeus has been described as an important species in industrial vinegar fermenters in central Europe (Sievers et al., 1992). The main biochemical differentiation characteristics of the A. europaeus isolates against the traditional Acetobacter species are both a strong tolerance to acetic acid, 4 to 8% in AE-agar and 10-14% in industrial fermentations, and requirement of acetic acid for growth. DNA homologies of industrial Acetobacter strains in the range of 30 to 100% with the A. europaeus type strain revealed their division into more than one group suggesting the occurrence of several new species or subspecies (Sievers et al., 1992).

Phenotypic and genotypic investigations of strains from industrial vinegar fermentations (cider- and spirit vinegar) and from a tea fungus beverage (Kombucha) made a group of Acetobacter strains stand out which grew in media with 3% acetic acid and 3% ethanol as does A. europaeus, did, however, not require acetic acid for growth. One Kombucha isolate (TF2) was chosen, its 16S rDNA and 23S rDNA (see chapter 3.2) were sequenced and DNA-DNA hybridizations with other Acetobacter and Gluconobacter strains were performed.

3.3.1 Phylogenetic positioning of Acetobacter intermedius

The 16S rRNA sequence of A. intermedius (EMBL accession number is Y14694) was determined by direct sequencing of the PCR-amplified rRNA gene product. The derived 16S rDNA primary structure was compared with homologous sequences taken from published databases including the sequences of all Acetobacter and Gluconobacter species previously described (Sievers et al., 1995). As shown in Table 17, the overall 16S rRNA nucleotide sequence similarities between A. europaeus, A. xylinus and A. intermedius are higher than 97% which made DNA pairing studies necessary as an additional genotypic parameter to underline the species status of A. intermedius (Stackebrandt and Goebel, 1994).

A phylogenetic tree reflecting the close relationships of the type strains is shown in Figure 15. The tree is based on the information contained in the 16S rRNA primary structures of the Acetobacter and Gluconobacter type strains and was constructed using the programs „pileup“, „distances“ and „growtree“ from the GCG software package. The very close clustering of A. europaeus, A. intermedius and A. xylinus only represents their present evolutionary status, their respective positioning in their branch of the phylogenetic tree is no proof for their species status (Ludwig W., 1998, personal communication). The species status of Acetobacter
intermedius is at this moment supported by DNA-DNA hybridization data and phenotypic properties like growth on AE-medium for all the strains, growth on GY-medium for 85% of the investigated strains and growth on MRS-medium for all the investigated strains (see chapter 3.3.4).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Ain</th>
<th>Aeu</th>
<th>Axy</th>
<th>Aha</th>
<th>Ali</th>
<th>Adi</th>
<th>Apa</th>
<th>Aac</th>
<th>Gox</th>
<th>Rgl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. intermedius</td>
<td>99.5</td>
<td>99.2</td>
<td>98.2</td>
<td>97.0</td>
<td>96.2</td>
<td>95.1</td>
<td>95.6</td>
<td>95.0</td>
<td>92.4</td>
<td></td>
</tr>
<tr>
<td>A. europaeus</td>
<td>99.5</td>
<td>98.4</td>
<td>97.4</td>
<td>97.0</td>
<td>95.1</td>
<td>95.4</td>
<td>94.8</td>
<td>93.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. xylinus</td>
<td>98.3</td>
<td>97.2</td>
<td>96.7</td>
<td>94.8</td>
<td>95.2</td>
<td>94.6</td>
<td>93.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. hansenii</td>
<td>97.1</td>
<td>97.0</td>
<td>94.5</td>
<td>94.2</td>
<td>94.4</td>
<td>93.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. liquefaciens</td>
<td>98.4</td>
<td>95.7</td>
<td>95.8</td>
<td>94.2</td>
<td>94.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. diazotrophicus</td>
<td></td>
<td>95.3</td>
<td>95.5</td>
<td>94.2</td>
<td>94.0</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. pasteurianus</td>
<td></td>
<td></td>
<td>96.8</td>
<td>95.0</td>
<td>92.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aceti</td>
<td></td>
<td></td>
<td></td>
<td>95.6</td>
<td>92.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. oxydans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>92.6</td>
<td></td>
</tr>
<tr>
<td>R. globiformis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 17. Overall 16S rRNA sequence similarity values for Acetobacter species, Gluconobacter oxydans and Rhodopila globiformis. Abbreviations: Ain, Acetobacter intermedius; Aeu, Acetobacter europaeus; Aac, Acetobacter aceti; Axy, Acetobacter xylinus; Aha, Acetobacter hansenii; Adi, Acetobacter diazotrophicus; Ali, Acetobacter liquefaciens; Apa, Acetobacter pasteurianus; Gox, Gluconobacter oxydans; Rgl, Rhodopila globiformis
Figure 15. Phylogenetic tree based on the 16S rRNA of all *Acetobacter* and *Gluconobacter* type strains. The tree was constructed with the GCG software package using the programs "pileup", "distances" (Jin-Nei Gamma) and "growtree" (UPGMA). The bar indicates 1% estimated sequence difference. Based on information from Sievers and Teuber, 1995.
3.3.2 DNA-DNA hybridization studies

The species status of *Acetobacter intermedius* can only be proven by DNA similarity experiments as its 16S and 23S *rrn* genes have homologies above 98% with the type strains of the species *A. europaeus* and *A. xylinus*.

Figure 16 shows the DNA-DNA hybridization of EcoRV-restricted and [α-32P]dATP-labeled *A. intermedius*<sup>T</sup>-DNA as a probe with the type strains of *A. europaeus*, *A. intermedius*, *A. xylinus*, *A. aceti*, *A. hansenii*, *A. liquefaciens*, *A. methanolicus*, *A. pasteurianus*, *Gluconobacter oxydans* and *Escherichia coli* HB101 and *Acetobacter* sp. strains from our laboratory. The highest homology of the *A. intermedius* type strain with all the type strains of *Acetobacter* and *Gluconobacter* was 60% with the type strain of *A. europaeus*. DNA-DNA hybridization of labeled *A. intermedius*<sup>T</sup>-DNA as a probe with other strains yielded the following results: 90% with strain JK2, 40% with strain V1, 65% with strain KO, 65% with TSA10, 99% with TSA8 and 90% with ØSSPR. Intense hybridization bands appear in the following strains in Figure 16: lane 3 (*G. oxydans*<sup>T</sup>), lane 9 (*A. xylinus*<sup>T</sup>), lane 11 (*A. intermedius*<sup>T</sup>), lane 12 (strain JK2) and lane 16 (strain TSA8). Such strong signals could be attributed to the occurrence of similar IS-elements in these strains. The intensities of the radioactive signals on the developed film were determined by quantitative densitometry.

The DNA-DNA hybridization values in Table 16 for the strains other than the *A. europaeus*, *A. intermedius* and the *A. xylinus* type strains were obtained by repeated hybridization experiments, the average of the obtained intensities of the hybridization signals was chosen as the result. The strains JK2 and V1 isolated from slovenic cider- and wine-vinegar are currently under investigation in a separate thesis (Trček, in preparation). Therefore, they are not further discussed in the present thesis.
Figure 16. DNA-DNA hybridization of EcoRV digested DNA with [α-\textsuperscript{32}P]dATP-labeled \textit{A. intermedius}\textsuperscript{T}-DNA as a probe. (A) Ethidium bromide stained chromosomal DNA pattern separated by electrophoresis. (B) Autoradiograph of chromosomal DNA pattern after hybridization with labeled \textit{A. intermedius}\textsuperscript{T}-DNA: lane 1, \textit{Escherichia coli} HB101; lane 2, \textit{Gluconobacter oxydans}\textsuperscript{T}; lane 3, \textit{A. aceti}\textsuperscript{T}; lane 4, \textit{A. pasteurianus}\textsuperscript{T}; lane 5, \textit{A. methanolicus}\textsuperscript{T}; lane 6, \textit{A. liquefaciens}\textsuperscript{T}; lane 7, \textit{A. diazotrophicus}\textsuperscript{T}; lane 8, \textit{A. hansenii}\textsuperscript{T}; lane 9, \textit{A. xylinus}\textsuperscript{T}; lane 10, \textit{A. europaeus}\textsuperscript{T}; lane 11, \textit{A. intermedius}\textsuperscript{T}; lane 12, \textit{A. europaeus} (JK2); lane 13, \textit{A. europaeus} (V1); lane 14, \textit{A. europaeus} (KO); lane 15, \textit{A. intermedius} (TSA10); lane 16, \textit{A. europaeus} (TSA8); lane 17, \textit{A. intermedius} (∅SSPR).
3.3.3 16S rDNA sequences of strains in phenon 3

The 16S rDNA oligonucleotide sequences of the *Acetobacter* strains from protein profile phenon 3 (Table 16) and strains JK3 and JKD were sequenced. Except for strain HE (identical sequence to *A. europaeus*) all 16S rDNA sequences were identical to that of *A. intermedius*.

3.3.4 Phenotypic characteristics

Phenotypic characteristics differentiating the *A. intermedius*- from the *A. europaeus-* and *A. xylinus-* type strains are shown in Table 18. The *A. intermedius* type strain grows with or without acetic acid, survives glucose concentrations of 20% both on media with and without 3% acetic acid and 3% ethanol and forms a cellulose pellicle on solid media and loose cellulose threads in liquid media which are sometimes suppressed. The *A. europaeus* type strain grows neither on GYG20% nor on AEG20%, does not form cellulose/acetan either in liquid or on solid media and requires acetic acid for growth and the *A. xylinus* type strain only grows on GYG20% and GY, cellulose formation is mostly present, in liquid media the production can spontaneously be suppressed.

Growth of *A. intermedius* and *A. europaeus* strains on AE, GY, AEG20%, GYG20%, selective MRS-medium for lactobacilli and on YP-media containing different carbon sources was tested. The growth of the cells on YP-media supplemented with different carbon sources gave no clear picture. Meaningful results on the use of carbon sources by the investigated strains would only be obtained in growth experiments on minimal media. As *A. europaeus*-strains generally need both acetic acid and an additional carbon source in their media, correct interpretation of results thus obtained would be tricky.

The *A. intermedius* strains all grow on AE-medium, 85% of the investigated *A. intermedius* strains grow on GY-medium and all the *A. intermedius* strains tested on MRS-agar showed excellent growth on this medium. These results clearly position *A. intermedius* as a new species with phenotypic properties of both *A. europaeus* and *A. xylinus*: *A. xylinus* strains do not tolerate concentrations of 3% acetic acid and the typical *A. europaeus* strain requires acetic acid for growth.

Classification of the strains grouped according to protein profile homology and DNA reassociation values in Table 16 as belonging either to *A. intermedius* or *A. europaeus* according to phenotypic properties is not always clear as some strains like strain SW3 (clearly established as *A. europaeus* by its DNA-homology value and its *rrnA* sequence) do exist that grow well on media without acetic acid (Table 19).
The *A. intermedius* type strain does not oxidize glucose to 5-ketogluconate or 2-ketogluconate. The production of either 5-ketogluconate or 2-ketogluconate or both is a variable trait (Kneubühl, 1994) and can therefore not be used for differentiation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>A. xylinus</th>
<th>A. europaeus</th>
<th>A. intermedius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on GYG20%</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on AEG20%</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth on AE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on GY</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on MRS</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GC content of DNA (mol%)</td>
<td>55-63</td>
<td>56-58</td>
<td>61.55</td>
</tr>
</tbody>
</table>

Table 18. Characteristics differentiating the type strains of *A. intermedius*, *A. xylinus* and *A. europaeus*. 
<table>
<thead>
<tr>
<th>strain</th>
<th>media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GYG20%</td>
</tr>
<tr>
<td>A. xylinus&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>A. europaeus&lt;sup&gt;T&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>KO</td>
<td>(+)</td>
</tr>
<tr>
<td>SW3</td>
<td>+</td>
</tr>
<tr>
<td>A. intermedius&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>JK3</td>
<td>+</td>
</tr>
<tr>
<td>JKD</td>
<td>+</td>
</tr>
<tr>
<td>TSN3</td>
<td>+</td>
</tr>
<tr>
<td>ØSSPR</td>
<td>+</td>
</tr>
<tr>
<td>TSN4</td>
<td>-</td>
</tr>
<tr>
<td>TSA10</td>
<td>(+)</td>
</tr>
<tr>
<td>E1</td>
<td>-</td>
</tr>
<tr>
<td>E2</td>
<td>+</td>
</tr>
<tr>
<td>Bodega</td>
<td>-</td>
</tr>
<tr>
<td>He</td>
<td>(+)</td>
</tr>
<tr>
<td>TSA7</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 19. Growth characteristics of different *A. europaeus*, *A. intermedius* and *A. xylinus* strains. Detailed description of the strains is on page 5 in Table 1. "-" indicates that no growth occurred on the media. "+" indicates that the strain grew on the media. "(+)" indicates that only little growth occurred on the media. Empty fields indicate that no experiment was performed.
3.3.5 Conclusion

Comparison of the 23S rRNA genes of the three closely related species *Acetobacter europaeus*, *Acetobacter intermedius* and *Acetobacter xylinus* confirmed the close genetic relatedness of > 98% of this molecule. The alignment of the 23S rDNA sequences revealed useful sites for the design of oligonucleotide probes. Hybridization signals of the oligonucleotide probe specific for the 23S rRNA of the *A. europaeus* type strain (23seu) were obtained with the *A. europaeus* type strain and *Acetobacter* strains from generators and acetators and also with three *Acetobacter* strains classified as *Acetobacter xylinus*. The oligonucleotide probe thus is not specific for *A. europaeus* strains. It is, however, a useful tool for the differentiation of *A. intermedius*. No strain of this species gave a positive hybridization signal with the oligonucleotide probe 23seu.

*Acetobacter intermedius* was isolated from a Kombucha beverage and has 16S rRNA and 23S rRNA homologies above 99% with *A. europaeus* and *A. xylinus*. Its DNA-DNA homology is <70% with *A. europaeus* and <30% with *A. xylinus*, and on the contrary to *A. europaeus* it shows growth on media both with and without acetic acid. These results confirm its species status. Differentiation of *A. europaeus* and *A. intermedius* strains solely on a phenotypic level is not suggested since *A. europaeus* strains without requirement for acetic acid for growth do exist.
4 Discussion

4.1 Plasmid pAEU601 of *Acetobacter europaeus*

The good detectability, the possibility of isolation of a usable quantity and its size make the 3.8 kb pAEU601 an interesting plasmid for further experiments to learn more about plasmid genetics in strains that produce high concentrations of acetic acid. Previous hybridization experiments (Kneubühl, 1994) with the plasmid extracted from the type strain of *Acetobacter europaeus* against the plasmids of *Acetobacter* strains used in the production of high acid vinegar gave strong signals indicating the distribution of similar plasmids throughout these strains. Strain TSA4 harbors a plasmid of the same size and copy number (decided according to intensity on gel electrophoretic picture) as pAEU601.

Experiments to construct a restriction map were not successful (Vela, 1995), many incomplete restrictions were obtained, one reason possibly being the methylation of a number of restriction sites in pAEU601. Transformation of pAEU601 into *E. coli* X-L1 Blue was not successful; with no known selection marker of pAEU601, selection of this plasmid was not possible (Vela, 1995). To rule out the possibility of incompatibility of pAEU601 and pUC18 (ColE1 replication type), hybridization of pAEU601 with the *in vitro* amplified oriV of pUC18 was performed. No signal was obtained. Jacsman, 1997, (Table 8) performed cloning experiments with *SalI* linearized plasmid and its two fragments obtained by *HindIII* restriction using high and low copy number plasmids and different competent cells. Only ligation of the large *HindIII* fragment of pAEU601 (RJ1) to pUC18 and transformation by electroporation into *E. coli* XL-1 Blue was successful. In a last try, the small *HindIII* fragment of pAEU601 (RJ3) was amplified *in vitro*, ligated to the vector pGEM®-T and successfully electroporated into *E.coli* XL-1 Blue. An explanation why ligation of RJ3 without prior amplification into pUC18 was not possible is difficult. The only complete ORFs in RJ3 are ORF2 with homologies to gyrase and mobilization proteins, no known interruptions of cloning experiments by these proteins are described, and ORF4 with no known coding function. *In vitro* amplification possibly could have changed the sequence of RJ3, also the higher quantity of DNA obtained with this method could have favorably influenced the cloning process.
So far, only one transformation experiment with a high acetic acid producing strain has been performed (Tayama et al., 1994): the not validly published strain „A. polyoxogenes“ (Entani et al., 1985) was transformed with plasmid DNA by electroporation in liquid media supplemented with antibiotics. Other transformation experiments of acetic acid bacteria with plasmid DNA were performed with strains with low tolerances to acetic acid such as A. aceti (Okumura et al., 1985), A. xylinus (Hall et al., 1992) and G. oxydans (Creaven et al., 1994).

4.1.1 Nucleotide sequence of plasmid pAEU601

The determination of the nucleotide sequence of pAEU601 provides the basis for examining the relationships between the DNA sequence and the gene organization of the plasmid. Comparison to the GenEMBL database revealed significant homologies with the plasmids pSC101 and RSF1010 (see chapter 3.1.5). For further examination those open reading frames which code for proteins with more than 75 amino acids and posses an appropriately placed Shine-Dalgarno sequence preceding the start codon (Table 9) were chosen as potential genes, translated in all three frames and compared to the available databases.

4.1.2 ORF1 and ORF2, genes possibly involved in mobilization of pAEU601

The ORFs 1 and 2 are divergently transcribed and are related to protein sequences of genes involved in mobilization of plasmids in bacteria.

ORF1 has 59% amino acid similarity and 40% identity (see Table 10) with the relaxation protein of plasmid pSC101 from Salmonella typhimurium (Bernardi et al., 1984) which, like ORF1, has significant similarities to both the mobilization protein MobL of the Thio|bacillus ferrooxidans plasmid pTF1 (protein accession number is P20085) and to the N-terminal domain of the mobilization protein MobA of the E. coli plasmid RSF1010 (protein accession number is P07112); the C-terminal domain of MobA has primase activity, is also expressed from an internal start site as a separate protein called RepB or B’ protein (Scholz et al., 1989) and could be involved in replication of RSF1010 (Scholz et al., 1989). Plasmid RSF1010 is well studied for its broad host-range replication properties among Gram-negative bacteria; Buchanan-Wollaston et al. (1987) proved that the mobilization functions of the RSF1010...
system are capable of transferring plasmids of *Agrobacterium* into plant cells. pSC101, on the other hand, has been used as the first cloning vector in recombinant DNA technology.

Formation of a nucleoprotein complex or relaxosome is the first step in the initiation of DNA transfer during bacterial conjugation (Guiney and Lanka, 1989). This initiation complex includes protein components capable of introducing a strand- and site-specific cleavage at a cognate oriT site on a supercoiled DNA substrate and generates a single stranded DNA to be transferred from donor to recipient cells (Drolet and Lau, 1992).

By aligning ORF1 on plasmid pAEU601 from *A. europaeus* to 6 other relaxation/mobilization proteins (4 of these proteins are listed in Table 10) with the program `eclustalw`, a conserved tyrosine at position 23 and two conserved histidines at positions 124 and 126 were identified. These highly conserved amino acids are part of conserved motifs found in proteins that nick supercoiled DNA. Ilyina and Koonin (1992) identified two amino acid motifs that that are conserved in two vast classes of proteins, one of which is involved in initiation and termination of rolling circle DNA replication and the other in mobilization of plasmid DNA. One motif consists of the sequence HisHydrHisHydrHydrHydrHydr, Hydr being a bulky hydrophobic residue, the other conserved motif encompasses a (putative) DNA-linking tyrosine residue. The motifs are differently oriented in the Mob and Rep proteins. Based on analogies with metalloenzymes, the authors hypothesized that the two conserved His residues were involved in metal ion coordination required for activity of the Rep and Mob proteins. In studies on the relaxase protein (TraI) of the conjugative plasmid RP4, Pansegrau *et al.* (1994) discovered three motifs in the TraI sequence that were conserved in relaxases from different origins. By site-directed mutagenesis of the RP4 traI gene the authors were able to show that tyrosine 22 (in motif I), aspartic acid 111 (in motif III), histidine 116 and histidine 118 (in motif III) are involved in oriT cleavage activity of the TraI protein. According to Pansegrau *et al.* (1994), the histidine 116 residue activates the aromatic hydroxyl group of tyrosine 22 by proton abstraction which leads to a transesterification reaction initiated by nucleophilic attack of the phosphodiester moiety at the nick site by the hydroxyl group of TraI tyrosine 22. The serine 74 residue in motif II is proposed to represent the DNA recognition domain of the enzyme.

As mentioned, the tyrosine of motif I and the HisHydrHisHydrHydrHydr motif of Ilyina and Koonin (1992) of motif III (Pansegrau *et al.* 1994) were identified in the amino acid sequence of ORF1. The amino acid sequence of ORF1 containing the two His residues of motif III is „Histidine Valine Histidine Methionine Methionine Phenylalanine“ with Valine, Methionine and
Phenylalanine as the hydrophobic residues (Figure 11). No conserved serine residue of motif II was discovered in the alignment of ORF1 with relaxation/mobilization proteins. The multiple alignment program was possibly run with inadequate gap parameters, as serine residues do appear in approximately the correct position in all sequences.

ORF2 has a 25% amino acid identity and a 43% similarity to the gyrase subunit B of *E. coli* (protein accession number is P06982) and *S. typhimurium* (protein accession number is Q60008). The protein gyrase belongs to the group II topoisomerases (topoisomerases are proteins that modulate supercoiling of DNA in cells). Gyrase adds negative supercoils to covalently closed circular double stranded DNA in an ATP-dependent manner. The protein is made up of two chains. Chain A is responsible for DNA breakage and rejoining, the B strain catalyzes ATP hydrolysis. The enzyme forms an A2B2 tetramer.

The amino acid sequence of ORF2 further has a 23% identity and a 47% similarity to the mobilization protein C of plasmid RSF1010 from *E. coli* (Scholz *et al.*, 1989 and Derbyshire *et al.*, 1987) and a 20% identity and 42% similarity to the mobilization protein S of plasmid pTF1 from *Thiobacillus ferrooxidans* (Drolet *et al.*, 1990).

The large and small sizes of ORFs 1 and 2 encoded by pAEU601, their sequence homology with the known Mob proteins of the RSF1010 and pTF1 plasmids and the similar arrangement to the pTF1 and RSF1010 *mob* genes support their involvement in possible mobilization of pAEU601. In Figure 17, the functional maps of the *mob* region of pTF1 and the region on pAEU601 encoding ORF1 and ORF2 are shown one above the other.
The components for the mobilization function of a plasmid DNA during conjugation include a *cis*-acting sequence (the origin of transfer, *oriT*) and a *trans*-acting sequence coding for mobilization proteins. Regions of sequence identity of plasmids pTF1, RSF1010 and pSC101 include their *oriT* sites which for pTF1 and RSF1010 reside in the intergenic space of the *mob* genes. The identified nick site in pTF1, which is adjacent to a characteristic 10 bp inverted repeat sequence, is also found for plasmid RSF1010 (Drolet *et al.*, 1990). No sequence homology to the intergenic region of ORFs 1 and 2 of pAEU601 is observed. Inverted repeat sequences do, however, appear in the intergenic region of ORFs 1 and 2 and are indicated in Figure 9 and Figure 17. Whether or not the nick site site of pAEU601 is also located in the intergenic region of ORF1 and ORF2 could be detected with the gel-electrophoretic assay using denaturing agarose gels developed by Pansegrau *et al.* (1988). Hereby the relaxed (OC) form of the plasmid is isolated and linearized with restriction enzymes that cleave it only once. Following alkaline denaturation into single strands, the DNA separates into three distinct
bands, one of which corresponds to the full linear length of the plasmid. The existence of two additional bands with higher mobility indicates site-specific relaxation of the plasmid, and the sizes of these fragments sum up to the full linear length.

A large portion of a self-transmissible plasmid is composed of the many *tra* genes involved in conjugation. Mobilizable plasmids lack these genes and their reliance on other plasmids allows them to be much smaller. The gene products of the *mob* region presumably include one gene that encodes a specific endonuclease (in relaxosome) that cleaves at the *oriT* site and another that encodes a helicase that displaces one strand (Snyder and Champness, 1997). Plasmid pAEU601 from the *Acetobacter europaeus* type strain is small (3.8kb). Its oligonucleotide sequence includes a region coding for two putative genes which are related to *mob* genes of other mobilizable plasmids. No *oriT* or *oriV* regions nor a gene that is possibly involved in replication has been identified. ORF2 does have 17% identity with the *rep* gene of plasmid pSC101 (Vocke and Bastia, 1983), for comparison studies in this work, however, only amino acid sequence identities above the delineation value of 20% to a known protein were taken into consideration. In plasmid RSF1010 of *E. coli* the *oriT* site is close to *oriV*. In this plasmid the C-terminal domain of MobA (primase activity) is also expressed from an internal start site as a separate protein called RepB or B' protein and it is suggested that some of the replication functions may play a dual role in mobilization (Scholz et al., 1989). ORF1 on pAEU601 from *A. europaeus* has a 33% identity only with the N-terminal part and not with the C-terminal part of protein MobA, this does not point to an involvement of the gene ORF1 encodes in replication of plasmid pAEU601. The replication modus of pAEU601 can not be described with the information found on the sequence sofar. A possibility to find the replicon of pAEU601 would be by restriction of the plasmid, ligation to a DNA fragment containing a selectable marker but without an *ori* region and transformation of bacterial cells with the recombinant DNA, selection would be for hybrids containing both the *ori* region of the plasmid and the piece of DNA with the marker gene (Snyder and Champness, 1997).

### 4.1.3 ORF3 and ORF7, similarities to virulence associated regions in bacteria

*Dichelobacter nodosus*, a strictly anaerobic Gram-negative rod, is the causative agent of ovine footrot. Labeled genomic DNA from the reference virulent strain A198 of *D. nodosus* and a benign isolate C305 were used to screen an A198 gene bank. One of the three recombinant plasmids which differentiated virulent and benign isolates of *D. nodosus* hybridized with all the
tested virulent and intermediate isolates of *D. nodosus*. The genomic region carried on this recombinant plasmid was designated as the virulence associated protein, or vap, region. Further chromosome walking experiments led to the cloning and delineation of the complete virulence associated locus, vrl, which encompassed ca. 25kbp (Rood *et al.*, 1994).

In Table 12 and chapter 3.1.10 the % identities and % similarities of ORFs 3 and 7 to amino acid sequences of proteins in the database Swissprot are shown. ORF3 has the highest similarity with a hypothetical protein (ORF1 from *Synechococcus* sp., described by Phung *et al.*, 1994). The vapA gene of *D. nodosus* is similar to the same partially-characterized ORF1 (Cheetham and Katz, 1995). The two authors have no explanation for these similarities. As the research on these virulence genes still leaves many open questions, the similarities of ORFs 3 and 7 of the plasmid pAEU601 to vap and vrl regions should for the moment be noted and not encourage one to jump to conclusions.

### 4.1.4 Conclusions

The plasmid pAEU601 of the *Acetobacter europaeus* type strain was chosen as a model molecule to analyse the gene structure of a plasmid from high acidic *Acetobacter* production strains. Problems arose when trying to clone the complete linearized plasmid and/or restriction fragments thereof. The two clones finally obtained were pRJ1 and pRJ3. pRJ1 is the large HindII fragment of pAEU601 ligated to pUC18, pRJ3 is the PCR amplified fragment of pAEU601 containing the small HindII fragment ligated to the pGEM®-T vector. Both clones contain the ori region for replication in *E. coli* and the gene coding for resistance to Ampicillin of pUC plasmids. Transformation experiments into *Acetobacter* and other bacteria still need to be performed.

The sequence of plasmid pAEU601 is a basis for more detailed molecular biology of *Acetobacter europaeus* and related strains. Plasmids of the same size and copy number as pAEU601 in other *Acetobacter* production strains have been described (Kneubühler, 1994; Sievers, personal communication). The homology of putative genes on pAEU601 with genes on other plasmids involved in mobilization suggests that plasmid pAEU601 may have been spread by mobilization. No nucleotide sequence homology with plasmids of other *Acetobacter* and *Gluconobacter* species was found.
4.2 *Acetobacter intermedius, sp.nov*

Compared to the 16S rRNA primary structure, the 23S rRNA molecule exhibits an increased degree of sequence variability (Stackebrandt, 1988) and has served as an appropriate target for probes to differentiate between genetically similar organisms (Rönner and Stackebrandt, 1994). Development of an oligonucleotide probe for *A. europaeus*-strains on the basis of 16S rDNA sequences was not possible due to the high similarity with *A. xylinus* (99.5%, see Table 17) with only 7 varying nucleotides distributed over the length of the gene. The alignment of the 23S rDNA sequences of the type strains of *A. europaeus*, *A. xylinus* and *A. intermedius* revealed equally high similarities (Table 13), however, with useful target sites for oligonucleotide probes. The oligonucleotide probe 23seu hybridized with all *Acetobacter* strains from the protein profile phenons 1, 2 and 4. It gave no hybridization signal with *A. intermedius*-strains, the *A. xylinus* type strain NCIB 11664 and the *A. xylinus* strain NCIB 613. However, it hybridized with three strains which are classified as *Acetobacter xylinus*: De Ley 25, ATCC 23768 and ATCC 10245. These strains have previously been studied for their DNA-DNA homologies with *A. europaeus* and *A. xylinus* (Boesch, 1994), all of them have DNA similarities below 30% with both the *A. europaeus* and the *A. xylinus* type strain. These results plus the results obtained in the hybridization studies with the oligonucleotide probe 23seu show that *A. europaeus*, *A. intermedius* and *A. xylinus* belong to a branch of rapidly evolving *Acetobacter* strains.

With the exception of one strain (HE, 16S rDNA identical to *A. europaeus*) all 16S rRNA genes of the strains from phenon 3 and two Slovenian strains that also did not hybridize with 23seu were identical to the 16S rDNA of *A. intermedius*. The overall 16S rRNA gene similarities between *A. europaeus*, *A. xylinus* and *A. intermedius* of more than 97% made DNA-DNA hybridization studies necessary as an additional genotypic parameter to underline the species status of *A. intermedius*. According to Wayne *et al.* (1987) a species would include strains with approximately 70% or greater DNA-DNA relatedness and which possess a common phenotypic characteristic. Previous DNA-DNA reassociation experiments with the *A. europaeus* type strain and all *Acetobacter* strains from the 4 phenons (Sievers and Teuber, 1995, Kneubühler, 1994 and unpublished results) had already resulted in the shifting of the strains of phenon 3 to subspecies level and grouping of the strains of phenon 4 in a possible new species.
DNA-DNA homology below the 70% threshold value for species delineation as well as three phenotypic differences (growth with or without acetic acid, growth on media containing 20% glucose, growth on MRS) clearly support the species status of the A. intermedius strains. Further DNA-DNA hybridization experiments with A. intermedius and A. europaeus strains (assigned according to their 16S rDNA sequences) with the A. intermedius type strain as a probe were performed more than once and produced the DNA-DNA homology values listed in Table 16. One strain whose data does not fit the these values is strain TSA8 from phenon 4 which shows less than 30% DNA homology with A. europaeus (Sievers and Teuber, 1995), hybridizes with the oligonucleotide probe 23seu and shows growth only on AE-medium, yet has a DNA-reassociation value of 99% with the A. intermedius type strain. The other strain whose data does not fit the values given in Table 16 is strain HE from phenon 3. This strain does not hybridize with the oligonucleotide probe 23seu, but its 16S rDNA sequence is identical to that of A. europaeus and its DNA-DNA homology with the A. intermedius type strain is <30%.

As mentioned, the delineation value for species level is 70% for DNA-DNA homology studies. The gathered results on A. europaeus, A. intermedius and A. xylinus (Sievers and Teuber, 1995; Kneubühler, 1994; Boesch, 1998) allow the grouping of the Acetobacter strains from phenon 3, strain TSA8 from phenon 4, strain TF2 (A. intermedius type strain) and strains JK3 and JKD from Slovenia in the new species Acetobacter intermedius. Although strains TSN1 and TSN5 hybridize with the oligonucleotide probe 23seu, their low DNA-DNA homology values to A. europaeus (<30%) position them in an as yet not defined and described species of Acetobacter. The oligonucleotide probe 23seu, is therefore only useful as a tool for the identification of Acetobacter species of the branch A. europaeus, A. intermedius, A. xylinus of the phylogenetic tree on page 57.

Bacterial isolates derived from a tea fungus on YPM (mannitol, peptone, yeast extract)- and AE-medium were previously classified to belong to the genus Acetobacter (Sievers et al., 1995). Based on the biosynthesis of cellulose, the results of the SDS-PAGE analysis and the growth on media without acetic acid, these isolates were tentatively identified as belonging to the species A. xylinus. The low clustering value of the A. xylinus type strain in the protein profile of 58% was attributed to the fact that this type strain is genotypically not thought to be representative for the species A. xylinus (unpublished results from our laboratory). Though
Discussion

these strains have been lost due to cultivation failure, the results based on the characterization of the newly isolated strain TF2 from the same Kombucha beverage indicate that the published strains (Sievers et al., 1995) would belong to the newly described species *A. intermedius*.

Yamada et al., 1997, have described the phylogenetic relationships of the acetic acid bacteria based on partial 16S rRNA gene sequences and propose the elevation of a subgenus *Gluconoacetobacter* to the generic level. It is generally accepted that only complete sequences allow reliable phylogenetic comparison with the available database of complete or almost complete sequences (Stackebrandt and Goebel, 1994). The construction of a phylogenetic tree based on partial base sequences must therefore be regarded as not valid. Also, one of the mentioned characteristics differentiating the 4 proposed genera, growth on mannitol agar only for the genera *Gluconobacter* and *Gluconoacetobacter*, is definitely wrong. In lists of bacterial cultures (Laboratorium Microbiologie Rijksuniversiteit Gent, BCCM Catalogue, 1989) YPM agar is suggested as medium for *Acetobacter* and *Gluconobacter* strains.

The main use of industrial strains is to produce high concentrations of acetic acid from ethanol. Industrial submerged fermenters are started by inoculation with 'seed vinegar' (microbiologically undefined remains from previous fermentations). Studies on plasmid profiles (Sievers and Teuber, 1995) clearly show that the transfer of the vinegar microflora from one fermenter to another is possible which suggests that industrially used strains have over the last two hundred years and more been adapted to an extreme niche and thus subjected to high evolutionary pressure. The close phenotypic and genotypic relationship of the three species *A. europaeus*, *A. intermedius* and *A. xylinus* would indicate that they belong to a rapidly evolving branch of the acetic acid bacteria as found in high acid products.

4.2.1 Description of the new species

*Acetobacter intermedius* (sp. nov.), L.adj.m. intermedius, in the middle between, to characterize the phenotype of this strain that has phenotypic properties of both *Acetobacter europaeus* and *Acetobacter xylinus*: growth in high concentrations of acetic acid and growth without acetic acid. Cells are Gram-negative, rod shaped, straight, 0.7μm by 2μm, occurring in pairs. Mobility and flagella not observed. Endospores are not formed. Obligately aerobic. Pale colonies, no pigments produced. Catalase positive. Oxidase negative. Oxidizes ethanol to
acetic acid. Acetate is oxidized to CO₂ but only below an acetate concentration of 6%. No oxidation of glucose to 5-ketogluconate or 2-ketogluconate. DNA base composition is 61.55%. *Acetobacter intermedius* occurs in tea fungus beverages, spirit vinegar and cider vinegar. No significant DNA-DNA hybridization with the type strains of *Acetobacter aceti*, *A. pasteurianus*, *A. liquefaciens*, *A. hansenii*, *A. xylinus*, *A. methanolicus*, and *Gluconobacter oxydans*. DNA-DNA hybridization with the *Acetobacter europaeus* type strain of <60%. Type strain: *Acetobacter intermedius* TF2 (DSM11804). Deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig. Isolated from a commercially available tea fungus beverage (Kombucha) in Switzerland.

### 4.2.2 Conclusions

Comparison and alignment of the 23S *rrna* genes of the three closely related strains *Acetobacter europaeus*, a high acetic acid production strain, *Acetobacter intermedius* from a Kombucha beverage and *Acetobacter xylinus* confirmed their close genetic relatedness and revealed useful sites for the design of oligonucleotide probes. Hybridization signals of the oligonucleotide probe specific for the 23S rRNA of the *A. europaeus* type strain (23seu) were obtained with the *A. europaeus* type strain and *Acetobacter* strains from acetators and generators but also with three *Acetobacter* strains classified as *Acetobacter xylinus*. For a complete and clear overview of the taxonomic situation, determination of the species status of previously classified *Acetobacter xylinus* strains needs to be done. The oligonucleotide probe 23seu is a useful tool for the differentiation of *A. intermedius* strains from *A. europaeus* and *A. xylinus*.

Based on 16S rDNA sequence alignments, missing hybridization signals with the oligonucleotide probe 23seu, DNA-DNA hybridization data and phenotypic properties like growth both on media with and without acetic acid and growth on MRS-agar, *Acetobacter intermedius* is described as a new species in the genus *Acetobacter*. 
5 References


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Born January 15, 1968 in Vienna, Austria

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