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16S-23S ribosomal RNA spacer regions of *Acetobacter europaeus* and *A. xylinum*, tRNA genes and antitermination sequences

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Abstract

The 16S-23S ribosomal RNA spacer regions of *Acetobacter europaeus* DSM 6160, *A. xylinum* NCIB 11664 and *A. xylinum* CL27 were amplified by PCR. Specific PCR products were obtained from each strain and their nucleotide sequences determined. The spacer region of *A. europaeus* comprises 768 nucleotides (nt), that of *A. xylinum* 778 nt and that of *A. xylinum* CL27 759 nt. Genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$ were identified. Putative antitermination sequences were found between the $tRNA^{Ala}$ sequence and the 5'-terminus of the 23S rRNA coding sequence. The boxA element has the nucleotide sequence TGCTCTTTGATA. Based on hybridization data of digested chromosomal DNA with spacer-specific probes, the copy number of the *rrn* operons on the chromosome of *Acetobacter* strains is estimated to be four.

Keywords: *Acetobacter*; Spacer region; tRNA; *rrn* operon; Antitermination

1. Introduction

The acetic acid bacteria, composed of the genera *Acetobacter* and *Gluconobacter*, represent together with *Rhodopila globiformis*, *Acidiphilium* and *Acidocella* a cluster of acidophilic bacteria with a distinct line of descent in the α-subclass of *Proteobacteria* [1,2]. The very closely related species *A. europaeus* and *A. xylinum* share a 99.6% 16S rRNA sequence similarity. The separate species status of these two organisms is based on DNA-DNA hybridization data and phenotypic differences such as the absolute requirement of acetic acid for the growth of *A. europaeus* [3]. *A. europaeus* strains have been isolated from industrial submerged vinegar fermentations carried out in acetators and trickle generators in Europe [4]. They are responsible for the production of vinegar with high acidity (7–14% acetic acid). *A. xylinum* is the archetype for the study of cellulose biogenesis [5,6]. Some strains of *A. xylinum* are components of the tea fungus producing the acidic kombucha beverage [7,8]. The ability of cellulose biosynthesis is often lost during cultivation of *A. europaeus*. In cider and wine vinegar production with acetic acid concentrations lower than 10%, *A. xylinum* cells can contaminate the vinegar plant via the substrate and cause fermentation problems due to their strong production of slime consisting of cellulose and acetan.

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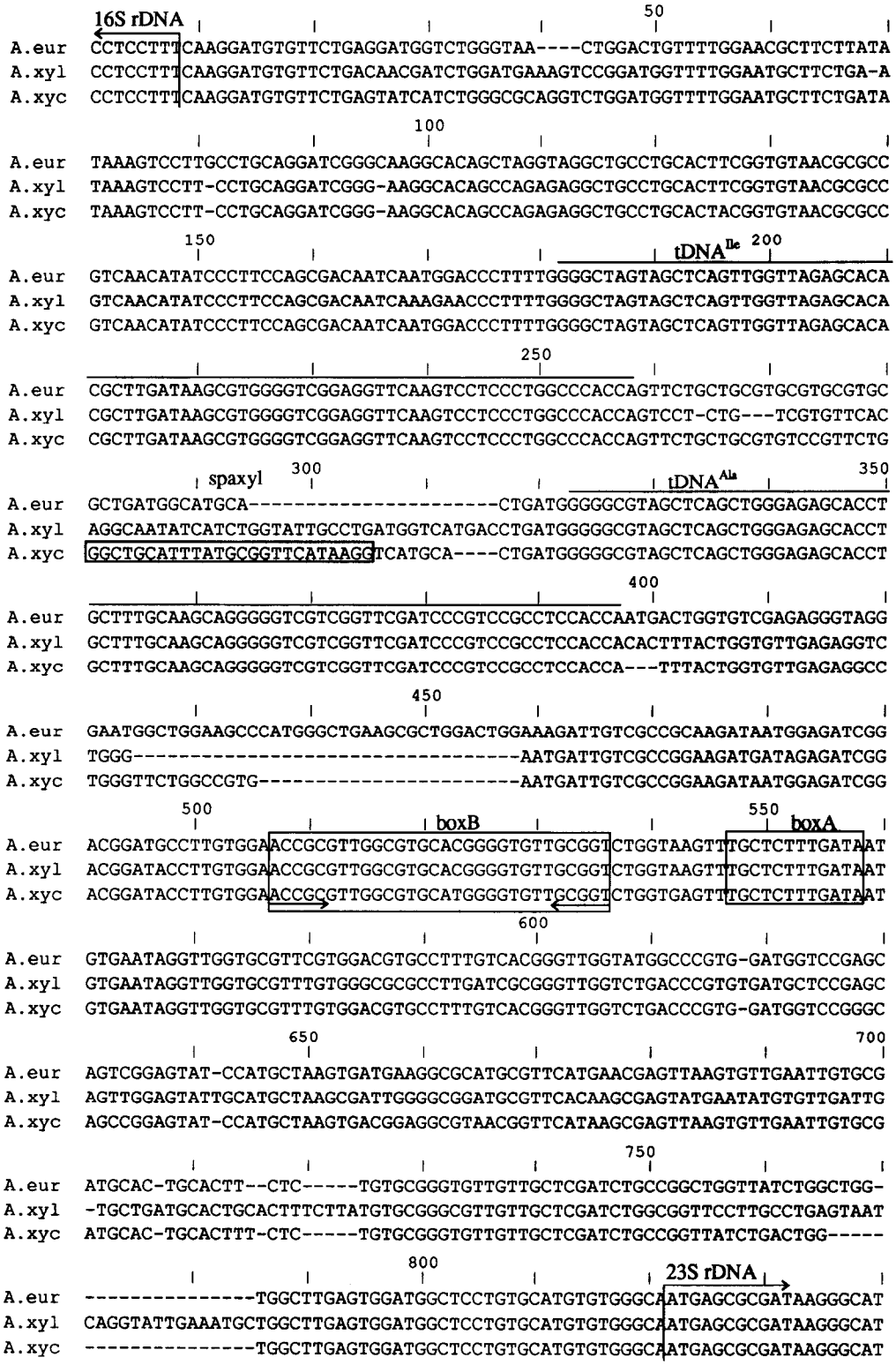
16S rDNA
50
A.eur CCTCCTTTCAAGGATGTGTTCTGAGGATGGTCTGGGTAA----CTGGACTGTTTTGGAACGCTTCTTATA
A.xyl CCTCCTTTCAAGGATGTGTTCTGACAACGATCTGGATGAAAGTCCGGATGGTTTTGGAATGCTTCTGA-A
A.xyc CCTCCTTTCAAGGATGTGTTCTGAGTATCATCTGGGCGCAGGTCTGGATGGTTTTGGAATGCTTCTGATA
100
A.eur TAAAGTCCTTGCCTGCAGGATCGGGCAAGGCACAGCTAGGTAGGCTGCCTGCACTTCGGTGTAACGCGCC
A.xyl TAAAGTCCTT-CCTGCAGGATCGGG-AAGGCACAGCCAGAGAGGCTGCCTGCACTTCGGTGTAACGCGCC
A.xyc TAAAGTCCTT-CCTGCAGGATCGGG-AAGGCACAGCCAGAGAGGCTGCCTGCACTACGGTGTAACGCGCC
150
tDNA^Ile
200
A.eur GTCAACATATCCCTTCCAGCGACAATCAATGGACCCTTTTGGGGCTAGTAGCTCAGTTGGTTAGAGCACA
A.xyl GTCAACATATCCCTTCCAGCGACAATCAAAGAACCCTTTTGGGGCTAGTAGCTCAGTTGGTTAGAGCACA
A.xyc GTCAACATATCCCTTCCAGCGACAATCAATGGACCCTTTTGGGGCTAGTAGCTCAGTTGGTTAGAGCACA
250
A.eur CGCTTGATAAGCGTGGGGTCGGAGGTTCAAGTCCTCCCTGGCCCACCAGTTCTGCTGCGTGCGTGCGTGC
A.xyl CGCTTGATAAGCGTGGGGTCGGAGGTTCAAGTCCTCCCTGGCCCACCAGTCCT-CTG---TCGTGTTCAC
A.xyc CGCTTGATAAGCGTGGGGTCGGAGGTTCAAGTCCTCCCTGGCCCACCAGTTCTGCTGCGTGTCCGTTCTG
spaxyl
300
tDNA^Ala
350
A.eur GCTGATGGCATGCA----------CTGATGGGGGCGTAGCTCAGCTGGGAGAGCACCT
A.xyl AGGCAATATCATCTGGTATTGCCTGATGGTCATGACCTGATGGGGGCGTAGCTCAGCTGGGAGAGCACCT
A.xyc GGCTGCATTTATGCGGTTCATAAGGTCATGCA----CTGATGGGGGCGTAGCTCAGCTGGGAGAGCACCT
400
A.eur GCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCCGCCTCCACCAATGACTGGTGTCGAGAGGGTAGG
A.xyl GCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCCGCCTCCACCACACTTTACTGGTGTTGAGAGGTC
A.xyc GCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGTCCGCCTCCACCA---TTTACTGGTGTTGAGAGGCC
450
A.eur GAATGGCTGGAAGCCCATGGGCTGAAGCGCTGGACTGGAAAGATTGTCGCCGCAAGATAATGGAGATCGG
A.xyl TGGG----------AATGATTGTCGCCGGAAGATGATAGAGATCGG
A.xyc TGGGTTCTGGCCGTG----------AATGATTGTCGCCGGAAGATAATGGAGATCGG
500
boxB
550
boxA
A.eur ACGGATGCCTTGTGGAACCGCGTTGGCGTGCACGGGGTGTTGCGGTCTGGTAAGTTTGCTCTTTGATAAT
A.xyl ACGGATACCTTGTGGAACCGCGTTGGCGTGCACGGGGTGTTGCGGTCTGGTAAGTTTGCTCTTTGATAAT
A.xyc ACGGATACCTTGTGGAACCGCGTTGGCGTGCATGGGGTGTTGCGGTCTGGTGAGTTTGCTCTTTGATAAT
600
A.eur GTGAATAGGTTGGTGCGTTCGTGGACGTGCCTTTGTCACGGGTTGGTATGGCCCGTG-GATGGTCCGAGC
A.xyl GTGAATAGGTTGGTGCGTTTGTGGGCGCGCCTTGATCGCGGGTTGGTCTGACCCGTGTGATGCTCCGAGC
A.xyc GTGAATAGGTTGGTGCGTTTGTGGACGTGCCTTTGTCACGGGTTGGTCTGACCCGTG-GATGGTCCGGGC
650
700
A.eur AGTCGGAGTAT-CCATGCTAAGTGATGAAGGCGCATGCGTTCATGAACGAGTTAAGTGTTGAATTGTGCG
A.xyl AGTTGGAGTATTGCATGCTAAGCGATTGGGGCGGATGCGTTCACAAGCGAGTATGAATATGTGTTGATTG
A.xyc AGCCGGAGTAT-CCATGCTAAGTGACGGAGGCGTAACGGTTCATAAGCGAGTTAAGTGTTGAATTGTGCG
750
A.eur ATGCAC-TGCACTT--CTC-----TGTGCGGGTGTTGTTGCTCGATCTGCCGGCTGGTTATCTGGCTGG-
A.xyl -TGCTGATGCACTGCACTTTCTTATGTGCGGGCGTTGTTGCTCGATCTGGCGGTTCCTTGCCTGAGTAAT
A.xyc ATGCAC-TGCACTTT-CTC-----TGTGCGGGTGTTGTTGCTCGATCTGCCGGTTATCTGACTGG-----
800
23S rDNA
A.eur ----------TGGCTTGAGTGGATGGCTCCTGTGCATGTGTGGGCAATGAGCGCGATAAGGGCAT
A.xyl CAGGTATTGAAATGCTGGCTTGAGTGGATGGCTCCTGTGCATGTGTGGGCAATGAGCGCGATAAGGGCAT
A.xyc ----------TGGCTTGAGTGGATGGCTCCTGTGCATGTGTGGGCAATGAGCGCGATAAGGGCAT

Fig. 1. Nucleotide sequence alignment of the 16S-23S spacer region of *Acetobacter europaeus* DSM 6160 (A.eur), *A. xylinum* NCIB 11664 (A.xyl) and *A. xylinum* CL 27 (A.xyc). DNA nucleotides corresponding to 16S and 23S rRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, spaxyl oligonucleotide probe and boxB-boxA elements are indicated. Inverted repeats are indicated by arrows under the nucleotides.
←

Ribosomal RNA molecules are powerful tools for the construction of phylogenetic trees and for the design of specific DNA and RNA probes for rapid identification of bacteria [9,10]. 16S-23S rRNA intergenic spacer regions are involved in the processing of ribosomal RNA transcripts by specific RNases [11] and promote antitermination of rRNA transcription [12].

In this study, the 16S-23S spacer regions of *A. europaeus* DSM 6160, *A. xylinum* NCIB 11664 and *A. xylinum* CL27 were sequenced. Based on these nucleotide sequences the type and structure of tRNA genes and antitermination sequences were determined, additionally, the number of distinct rRNA operons on the chromosomes were estimated.

2. Material and methods

2.1. Bacterial strains

A. europaeus DSM 6160 was cultivated in a medium containing 0.2% yeast extract, 0.3% peptone, 0.5% glucose, 3.0% acetic acid and 3.0% ethanol. *A. xylinum* NCIB 11664 and *A. xylinum* CL27 were grown in a medium containing 5.0% glucose, 1.0% yeast extract and 1.0% $CaCO_3$. Cultivation was at 30°C on a rotatory shaker to the late exponential phase. *A. europaeus* was isolated from a submerged vinegar fermentation process [4], *A. xylinum* NCIB 11664 was obtained from the LMG culture collection (Gent, Belgium) and *A. xylinum* CL27 was isolated from a tea fungus fermentation [8].

2.2. Identification methods

Biochemical and physiological characteristics were investigated as reported previously [4].

2.3. In vitro amplification and cloning of the 16S-23S intergenic spacer region

PCR was performed as described by Both et al. [13]. Primers used for amplification of the spacer region were A16: 5′-TTGCATGGATCCTGCGGCTGGATCACCTCC-3′ and B23: 5′-GAATCAGGATCCGAATGCCCTTATCGCGCTC-3′. The primers contain *Bam*HI recognition sites to facilitate subsequent ligation with the vector pUC18. After initial denaturation of the DNA, reaction conditions were: 92°C for 1 min, 57°C for 2 min, 72°C for 2 min with 35 cycles. The vector pUC18 was used as vehicle for cloning of the purified spacer fragments. The DNA was transferred into *E. coli* XL1-Blue (Stratagene, La Jolla, CA) by electro-transformation. The recombinant DNA was extracted and purified by gradient centrifugation [14].

2.4. Sequencing

The nucleotide sequence of the cloned spacer region was determined by applying the chain termination method using the Sequenase version 2.0 DNA sequencing kit (US Biochemicals) in combination with [α-^{35}S]dATP. The spacer sequences were obtained by the use of specific pUC primers (reverse primer and −40 primer) and by the strategy of primer walking.

2.5. DNA-DNA hybridization

Digested DNA of *A. xylinum* CL27 was transferred from 0.8% agarose gels to positively charged nylon membranes by vacuum blotting. The [α-^{32}P]-dATP labelled spacer PCR fragment of *A. xylinum* CL27 was used as hybridization probe. The hybridization was carried out at 65°C under stringent conditions. An oligonucleotide probe, named spaxyl, with the sequence GGCTGCATTTATGCGGTTCATAAGG derived from the spacer sequence of the chromosome of *A. xylinum* CL27 was [γ-^{32}P]-dATP labelled with T4 polynucleotide kinase and the hybridization was carried out at 48°C under optimal conditions (T_m−26°C). Southern hybridization was performed as described by Sambrook et al. [14].

2.6. Nucleotide sequence accession number

The nucleotide sequences of the 16S-23S intergenic spacer regions of *A. europaeus* DSM 6160, *A. xylinum* NCIB 11664 and *A. xylinum* CL27 have been deposited with EMBL (Cambridge, UK) under the accession numbers X85406, X85405 and X85726, respectively.

3. Results

3.1. Sequences and general features of the spacer region

The 16S-23S intergenic spacer regions from *A. europaeus* DSM 6160, *A. xylinum* NCIB 11664 and *A. xylinum* CL27 were amplified and their nucleotide sequences were determined. The first primer A16 used for PCR is complementary to *E. coli* 16S rRNA sequence positions 1520–1539 and primer B23 is complementary to *E. coli* 23S rRNA sequence positions 3–24. The resulting PCR products of the three strains were analyzed on a 0.8% (w/v) agarose gel. One distinct band, corresponding to 802 bp in length for *A. europaeus* DSM 6160, 812 bp for *A. xylinum* NCIB 11664 and 793 bp for *A. xylinum* CL27 was identified after amplification. Enzymatic restriction of the single PCR fragment revealed *Pvu*II fragments in the size of 300 and 500 bp for *A. europaeus*, 250 and 550 bp *Hae*III fragments for *A. xylinum* and 300 and 500 bp *Eco*RV fragments for *A. xylinum* CL27, respectively. In each case the sum of the restriction fragments adds up to the size of the original PCR fragment. The spacer regions of *A. europaeus* and *A. xylinum* contain no recognition site for *Eco*RV.

The alignment of DNA sequences of the 16S-23S intergenic spacer regions of the three strains is shown in Fig. 1. The spacer region of *A. europaeus* comprises 768 nt, of *A. xylinum* 778 nt and of *A. xylinum* CL27 759 nt. Comparison between these three sequences shows different stretches of conserved, variable and hypervariable character. Two regions in each spacer sequence were homologous to tRNA genes. 77 nucleotides from the sequences that span positions 182–258 in our coordinates are coincident with those of $tRNA^{Ile}$ and 76 nucleotides that span

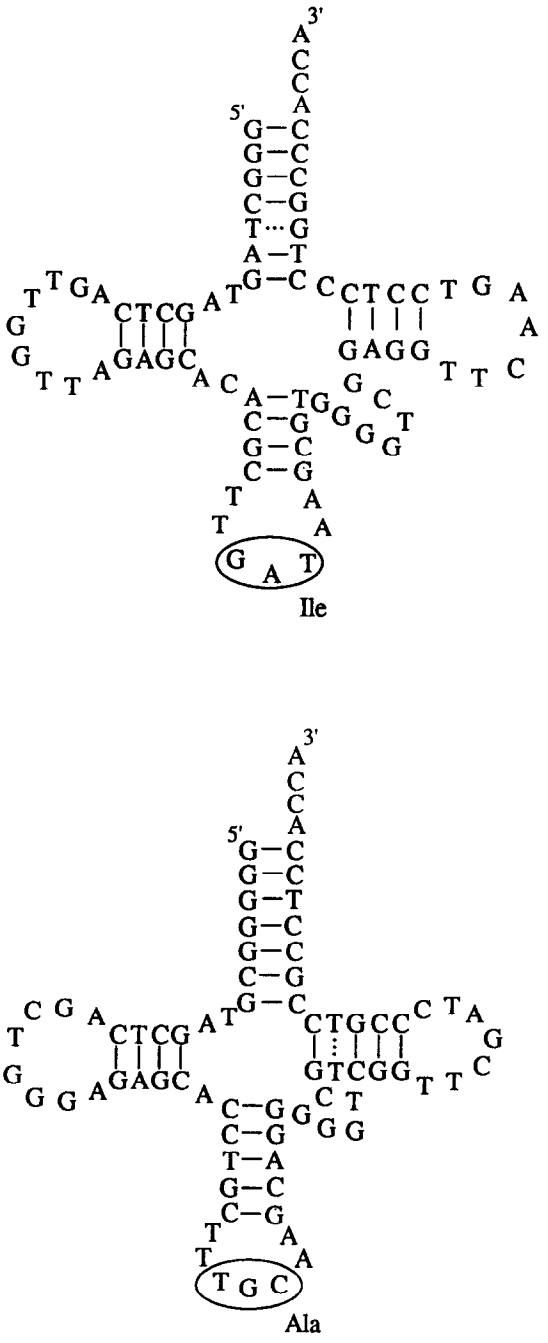

Fig. 2. Secondary structure of the sequences coding for tRNA Isoleucin (Ile) and Alanin (Ala) molecules in the 16S-23S spacer regions of *Acetobacter europaeus* and *A. xylinum*. Nucleotides that correspond to anticodons are framed. The noncanonical T:G and T:T base pairs are marked by dots.

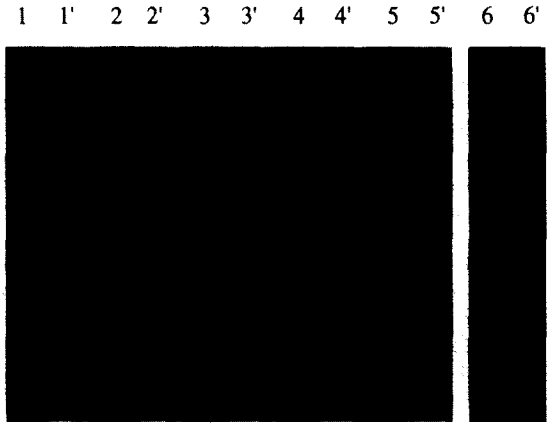

Fig. 3. Southern hybridization of *Acetobacter xylinum* CL27 DNA digested with: lane 1, *Pvu*I; lane 2, *Mlu*I; lane 3, *Acc*I; lane 4, *Cla*I/*Mlu*I; lane 5, *Pvu*I/*Mlu*I; lane 6, *Hpa*I. Fragmented DNA was resolved by agarose gel electrophoresis and transferred to Nylon membranes. Hybridization of transferred DNA with the (α-^{32}P)-labelled PCR fragment of the 16S-23S spacer region of *A. xylinum* CL27 is shown on lanes 1′–5′. Hybridization with the (γ-^{32}P)-labelled spaxyl oligonucleotide probe is shown on lane 6′.

positions 323–397 correspond to tRNA^{Ala}. The tRNA^{Ile} and tRNA^{Ala} sequences are identical in all strains. They are separated by 42 nt in *A. europaeus*, by 60 nt in *A. xylinum* and by 60 nt in *A. xylinum* CL27. Fig. 2 shows the proposed secondary structure models of tRNA^{Ile} and tRNA^{Ala} derived from the corresponding sequences in the investigated spacer regions. The D-arm of tRNA^{Ile} consists of four base pairs and eight nucleotides forming a loop. The TΨC-arm of tRNA^{Ile} consists of four base pairs and seven nucleotides forming a loop. The tRNA^{Ala} D-arm contains four base pairs and eight nucleotides forming a loop, the TΨC-arm of the molecule consists of five base pairs with one noncanonical pair (T:T) and seven nucleotides forming a loop.

To prevent premature transcription, rRNA operons usually are equipped with antitermination elements. Downstream from the tRNA^{Ala} gene a boxA with the nucleotide sequence TGCTCTTTGATA and a putative boxB consisting of 30 nucleotides with short inverted repeats are present in the spacer region of the investigated strains (Fig. 1). Comparison between the boxA and boxB elements present in *A. europaeus* and *A. xylinum* revealed identical nucleotide sequences. No promotor sequences upstream from the boxA and boxB elements were found in the spacer region of the investigated *Acetobacter* strains by computational analysis.

3.2. Copy number of the spacer regions

To determine the copy number of the spacer regions in the chromosomes of the investigated *Acetobacter* strains, we carried out Southern hybridization analyses with restriction digests of chromosomal DNA of *A. xylinum* CL27 using the homologous PCR fragment as a probe. Based on single digests with *Pvu*I, *Mlu*I, *Acc*I and with double digests of *Cla*I/*Mlu*I as well as *Pvu*I/*Mlu*I, four distinct bands were obtained for each restriction digest on the corresponding autoradiogram of the Southern hybridization (Fig. 3). Since the restriction enzymes used in this study do not cut in the PCR fragment, the copy number of the spacer region is estimated to be four. This finding is supported by a hybridization analysis with an oligonucleotide probe, named spaxyl, derived from the spacer region of *A. xylinum* CL27 (Fig. 1). Single digestion with the restriction enzyme *Hpa*I of the genomic DNA of this strain results in four bands on the corresponding autoradiogram after hybridization with the spaxyl probe (Fig. 3, lanes 6 and 6′). Four equally distinct bands also appeared on the corresponding autoradiogram by hybridization of digested chromosomal DNA (*Bst*EII and *Mlu*I) of *A. xylinum* NCIB 11664 with the homologous PCR fragment. Analogous hybridization data were obtained with *Bcl*I- and *Bam*HI/*Hin*dIII-digested chromosomal DNA of *A. europaeus*.

4. Discussion

PCR of the 16S and 23S RNA intergenic spacer region from the chromosomal DNA of *A. europaeus* DSM 6160, *A. xylinum* NCIB 11664 and *A. xylinum* CL27 produced one precise amplification product for each strain. Sequences coding for tRNA^{Ile} and tRNA^{Ala} were detected in the spacer regions. Each type of tRNA is identical in the three strains. Sequence homology of the tRNA genes in the spacer region between *Acetobacter* and *E. coli* [15], *Rhodobacter sphaeroides* [16], *Thiobacillus ferrooxidans* [17], *Bacillus subtilis* [18] and *Acholeplasma laidlawii* [19]

is 84.0, 87.0, 83.3, 85.7 and 87.0%, respectively for $tRNA^{Ile}$ and 82.9, 90.8, 86.8, 85.5 and 82.7%, respectively for $tRNA^{Ala}$. Sequence comparison of the tRNA genes between different members of the domain *Bacteria* clearly demonstrates the functional conservation of these molecules. Transcription of one of the seven noncontiguous ribosomal rRNA operons (rrn A-E and rrn G-H) from *E. coli* proceeds as a 30S precursor rRNA. Antitermination systems in ribosomal RNA genes play a necessary role in avoiding premature termination of the transcription product [12]. Antitermination elements have been detected in regions upstream of 16S genes as well as in 16S-23S spacer regions. The spacer element, boxA, of the antitermination system is a conserved sequence (TGCTCTTTAACA), while the leader element, boxB, is a highly variable sequence [12]. The boxA element we found has two mismatches with the consensus sequence. The putative boxB element in the spacer region of *Acetobacter* is proposed to form a stem-loop structure with the inverted repeats consisting of five nucleotides.

Based on the results from the hybridization data with single and double restriction digests, we propose that four copies of the spacer region are present on the chromosome of *A. xylinum* and *A. europaeus*. From the PCR amplification and restriction enzyme profile we concluded that the sequences of the spacer regions of the four ribosomal RNA operons present within one *Acetobacter* strain are identical.

Differences in the nucleotide sequence of the spacer region and 23S rRNA enable the development of specific DNA probes for discrimination between *A. europaeus* and *A. xylinum* strains on the DNA level.

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