Phylogenetic Analysis of 16S rDNA Sequences of *Pediococcus acidilactici* TISTR 2309: Relationships between Closely Related Species

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Abstract
Phylogeny is the study of relationships and evolution between collections of things (genes, proteins, organisms or etc.) that are derived from a common ancestor. It is a useful tool for classifying organisms and gives better results than traditional identification methods based on morphological, metabolic and other phenotypic traits. *Pediococcus acidilactici* TISTR 2309 is one of the lactic acid bacteria (LAB) that was found in traditional Thai fermented sausages and it has the potential to produce bacteriocin against foodborne Gram positive bacteria, including *Bacillus cereus* and *Staphylococcus aureus*. Three phylogenetic trees derived from the Maximum Likelihood, Neighbor-Joining and Maximum Pasimony methods, were compared in order to find the relationship of the strains constructed by each method. The results from phylogenetic analysis of 16S rDNA sequences by these 3 methods indicated that *P. acidilactici* TISTR 2309 is homogenous to *P. acidilactici* IMAU60189. However when the same sequence of TISTR 2309 was used as input for search tools of NCBI Blast and ENA, the two databases provided different results of identification and different suggested strains. It was also found that the potential in bacteriocin production of the *P. acidilactici* TISTR 2309 isolate did not reduce over nine years of laboratory subculture.

Keywords: Lactic acid bacteria, *Pediococcus acidilactici*, Bacteriocin, Phylogeny, Cladogram, 16S rDNA

1 Introduction

For more than a century, organisms have been classified as species based on the similarity of morphological features and ecology. Taxonomy is the science for description, identification, nomenclature and classification of organisms. It also enables us to understand the evolutionary history of life on earth. If two taxa are more similar then they are regarded as more closely related. By looking at the differences and similarities between organisms, biologists can construct an evolutionary tree which is called phylogeny [1]. Phylogeny is considered as a branch of taxonomy which studies the relationships and evolution between collections of biological entities (genes, proteomes, transcriptomes, etc.) that are derived from a common ancestor. At present, phylogenetic trees based on nucleic acid sequences are widely accepted as a method for classifying and identifying bacteria which gives more reliable results than the conventional approaches based on morphological, metabolic and other phenotypic traits [2]–[4].

There are 2 major methods to build phylogenic trees [5], [6].

1. Clustering methods are based on statistical methods for determination of phylogenetic relationships and they are explicitly nonhistorical. The methods are also recognized as a phenotype approach which

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basically depends on similarity between organisms. A popular clustering method, known as the unweight pair group method with arithmetic mean (UPGMA). is usually used to construct trees based on DNA or protein sequence data. The Neighbour-Joining method is a modified UPGMA method which is designed to correct for unequal rates of evolution in different branches of a tree [5].

2. Cladistic methods select a tree by utilizing an explicit model of the evolutionary process, and thus depend on genealogy. The most popular cladistic methods in molecular phylogeny are the maximum parsimony and maximum likelihood methods. The maximum parsimony method is a particular non-parametric statistical method for constructing phylogenies by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length. Whereas the maximum likelihood computes to find the most possible topology of the evolutionary tree [5].

The ribosome of prokaryotes is composed of 3 ribosomal RNAs, called the 5S, 16S and 23S rRNA, which are typically organized as a co-transcribed operon (Table 1). These rRNAs are produced from the structural DNA in the chromosome which is known as the rDNA. The rDNA genes, particularly 16s rDNA, become an alternative way to be exploited in phylogenetic analysis for several reasons. Firstly, they are present in almost all bacteria, often existing as a multigene family, or operons. Secondly, the function of the 16S rDNA gene has not changed over time and it is a highly conserved sequence. This means that sequences from distantly related organisms can be precisely aligned and are easy to measure. Lastly, and very importantly, the 16S rDNA gene (1,500 bp) is large enough for informatic purposes [7]. Therefore, comparison of 16S rDNA sequences is an effective method for showing relationships of evolution among microorganisms [2].

Table 1: Ribosomal RNAs in prokaryotes

Name	Size (nucleotides)	Location
5S*	120	Large subunit of ribosome
16S*	1500	Small subunit of ribosome
23S*	2900	Large subunit of ribosome

*S = Svedberg units

Lactic acid bacteria (LAB) are Gram positive bacteria which are commonly found in vegetables, fruits and meat. LAB have been used in the food industry for the production, preservation and fermentation of a wide variety of food and diary products [8]–[10]. There are many publications that report studies of the evolution of LAB of the families *Bacillaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae* in the order of *Lactobacillales* and especially of the genus *Lactobacillus* [2], [11]–[14]. However, there are few publications that report studies of the genus *Pediococcus*. *Pediococcus acidilactici* is one of the species of lactic acid bacteria which is used in the food industry for application as starter culture for fermentation of meat and vegetable products [15].

The optimum conditions for the growth of *P. acidilactici* is 40°C, at pH 6.0 [16]. Some strains of *P. acidlactici* have been reported to produce bacteriocin. For instance pediocin PA-1 was obtained from *P. acidlactici* PAC-1 [17], pediocin AcM was harvested from *P. acidilactici* M [18], and pediocin SA-1 was purified from *P. acidilactici* NRRL B5627 [19].

This study focuses on our isolate, *P. acidilactici*, which was originally isolated from Mum (traditional Thai fermented sausage) in the year 2006 [9] and has been given the name of strain CP7-3. Previous work [9], [16] has shown that the strain produced a bacteriocinogenic substance which inhibited the growth of foodborne Gram positive bacteria such as *Bacillus cereus* TISTR 037 and *Staphylococcus aureus* TISTR 029. Recently, the culture of *P. acidilactici* CP 7-3 was sent to the Thailand Institute of Scientific and Technological Research (TISTR) culture collection for preservation and was officially named as *P. acidilactici* TISTR 2309.

This article aims to compare the relationship of *P. acidilactici* TISTR 2309 with related strains of LAB using three different cladogram methods, namely, Maximum Likelihood, Neighbor-Joining and Maximum Parsimony. The 16S rDNA sequence of the isolate was compared with other strains which have a % identity close to the isolate as well as with an outgroup species and phylogenetic trees were then constructed for the strains using each of the three cladogram methods. The bacteriocin producing properties between the freshly isolate, *P. acidilactici* CP 7-3 [9], 3 years culturing [16] and the same isolate after 9 years of maintaining *P. acidilactici* TISTR 2309 (this study) were analyzed. The differentiation by time of *P. acidilactici* CP 7-3 [9] and *P. acidilactici*

TISTR 2309 was studied by comparing the changes of 16S rDNA sequence.

2 Methods

2.1 *Microorganisms and medium*

Pediococcus acidilactici TISTR 2309 was grown in MRS broth at 37°C in 5% CO_2 for 24 h, maintained at 4°C after growth and sub-cultured every month. For stock culture, the bacterium was stored in a liquid medium containing 20% (v/v) glycerol at –196°C. For test organisms, *Bacillus cereus* TISTR 037 and *Staphylococcus aureus* TISTR 029 were cultivated in nutrient agar at 37°C for 24 h prior to use.

2.2 *Sequencing of 16S rDNA gene analysis*

The sequence analysis of *P. acidilactici* TISTR 2309 (including PCR amplification, direct sequencing of 16S rDNA and sequence analyses) was performed by BIOTEC culture collection, National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand. The primers and the methods used in preparing the isolates used in this study and in the previous study in reference [9] are shown in Table 2.

2.3 *Sequence identification*

The full length 16S rDNA sequence of *P. acidilactici* TISTR 2309 was entered in the BLAST webpages of the National Center for Biotechnology Information (NCBI) [20] and the European Nucleotide Archive (ENA) [21]. The results of suggested identifications from the websites which provided more than 93% identity were then selected and the comparative 16S rDNA sequences were downloaded from the database.

2.4 *Phylogenetic analysis*

The full length 16S rDNA sequence of *P. acidilactici* TISTR 2309 was compared with 10 of 16S rDNA sequences of *Pediococcus* spp., 2 of 16S rDNA sequences of *Lactobacillus* spp. (Table 3) and 1 of 16S rDNA sequence of *E. coli* as an outgroup. All chosen sequences were in the range of 1400 to 1500 bases. These sequences were obtained from available LAB genomes in both ENA [21] and in the genome section of NCBI databases [20]. The Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 with the maximum parsimony, maximum likelihood, and neighbour-joining methods were used to construct phylogenetic trees of those sequences.

Table 2: Microorganisms, primer and method for sequencing of 16S rDNA

Microorganisms	Forward primer	Reverse primer	Method	References
P. acidilactici TISTR 2309	518F (5'-CCAGCAGC CGCGGTAATACG-3')	1429R (5'-TACGGYTAC CTTGTTACGACTT-3')	Full length 16S rDNA analysis by using ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit V3.1	This study
P. acidilactici TISTR 2309 (called *CP* 7-3 in previous study)	UFUL (5'-GCCTAACA CATGCAAGTCGA-3')	URUL (5'-CGTATTAC CGCGGCTGCTGG-3')	Partial sequence of 16S rDNA analysis by using Bigdye V.3.1 cycle sequencing kit	[9]

Table 3: Comparison of %identities retrieved from NCBI and ENA

Source	Microorganisms	Accession No.	%identities
NCBI [18]	*Pediococcus acidilactici* DSM20284	NZ GL397069.1	99.58
	Pediococcus lolii NGRI0510Q	BANK 01000001	99.36
	Pediococcus pentosaceus ATCC 25745	NC 008525.1	98.00
	Lactobacillus brevis ATCC 367	NC 008497.1	93.00
	Lactobacillus reuteri DSM 20016	NC 0095513.1	93.00
ENA [17]	*Pediococcus acidilactici* JFP1	EM_PRO:KM062019	99.70
	Pediococcus lolii LMG25667	EM_PRO:JX311435	99.70
	Pediococcus lolii LMG27029	EM_PRO:JX311434	99.70
	Pediococcus acidilactici IMAU60189	EM_PRO:J917739	99.70
	Pediococcus acidilactici IMAU20070	EM_PRO:FJ844982	99.70
	Pediococcus acidilactici IMAU10073	EM_PRO:FJ915729	99.70
	Pediococcus acidilactici BFE 8246	EM_PRO:EU147310	99.70

Nucleotide region of	Nucleotide sequence (5' -> 3')
16S rDNA	>CP7-3 / TISTR 2309 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTTCCGTTAATTGATTATGACGTGCTTGCACTGAATGAGA TTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACA GATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCG CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACAT TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCA ACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACC CAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGCGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTCATCC GGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCAT TGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAA CACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACC CTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTNGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAG TAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGCCAACCTAAGAGATTAGGCGTTCCCTTCGGG GACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC TTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTC AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAAACCGCGAGGTTTA GCTAATCTCTTAAAACCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCG GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCGCACCATGAGAGTTTGTAACACCCAAAG

Figure 1: The full length 16S rDNA sequence of *P. acidilactici* TISTR 2309.

2.5 *Bacteriocin activity assay*

P. acidilactici TISTR 2309 was sub-cultured in Tryptic Soy Agar (TSA) without glucose (Becton, France) plus 2.0% yeast extract (Himedia, India) at 37°C for 24 hours in CO_2 incubator (Hotpack, USA). The bacterial colonies were suspended in normal saline solution (0.85% (w/v) NaCl) at the initial absorbance (600 nm) of 0.5 and then used as inoculum for bacteriocin production. 2% (v/v) of suspended cells were inoculated into Tryptic Soy Broth (glucose excluded) (Becton, France) added with 2.0% yeast extract (TSBYE) [22]. TSB (without glucose) was used in order to avoid lactic acid production and the final pH was maintained at 6.0 [16]. The culture was then incubated at 37°C for 24 h in CO_2 incubator (Hotpack, USA), without agitation. To harvest the crude bacteriocin, the culture was centrifuged at 10,000 G (Tomy, Japan), 4°C for 15 minutes. Then, supernatant was filtered through 0.22 µm sterile cellulose acetate membrane. The cell free solution was tested for bacteriocin activity using a modified microtiter plate assay [23]. Each indicator bacteria was suspended in normal saline solution to an $OD_{600\,nm}$ of 0.5. The assay consisted of 50 µl suspended test bacteria mixed with an equal volume of the filtrate and 100 µl of Mueller Hinton Broth (MHB, Himedia, India). After incubation at 37°C for 24h in a 96-well plate, the turbidity of the solution was measured by a microplate reader (BIORAD) at $OD_{600\,nm}$.

One bacteriocin unit (B.U.) was defined to be the amount of bacteriocin which inhibits the growth of the indicator bacteria by 50% when compared with a bacteriocin-free control with TSBYE filtrate, i.e., B.U./ ml = 50% inhibition / dilution factor.

3 Results and discussion

3.1 *The full 16s rDNA sequencing and phylogenetic analysis*

The full 16S rDNA sequence of *P. acidilactici* TISTR 2309 comprised 1440 nucleotides (Figure 1). The sequence data were submitted to GenBank® [24] on July 2015. The given accession number is KT265690.

3.2 *Blast searches of P. acidilactici TISTR 2309*

The characteristics of *P. acidilactici* TISTR 2309 have been reported previously as a Gram-positive, coccus, found in pairs or tetrads, homo-fermentative, facultative anaerobes which grow in wide ranges of pH and temperature [9]. When the nucleotide sequences were entered in the search query of the NCBI and ENA databases [20], [21], the suggested strains that exhibited highest percentage of similarity are shown in Table 3. The results obtained from the two sources were different. Although the NCBI database is the most popular among molecular scientists, the results showed that the highest match gave only 99.58% identity to *P. acidilactici* DSM20284. In contrast, the ENA displayed the highest matching identity of 99.7% to 7 different strains of *Pediococcus* spp. On checking, we found that the NCBI database did not contain the 7 strains of the *Pediococcus* spp. matched with TISTR 2309 by the ENA database and that the ENA database did not include the *P. acidilactici* DSM20284 matched by the NCBI database. According to the literature, the GenBank database, maintained by the NCBI, exchanges information with the ENA and the DNA

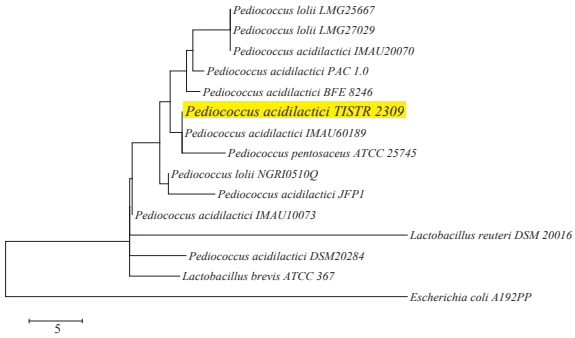

Figure 2: Phylogenetic tree based on 16s rDNA of *P. acidilactici* TISTR 2309 compared with other strains based on the maximum likelihood method. The scale bar represents the number of substitutions per site. The tree was constructed with MEGA v6.0 software.

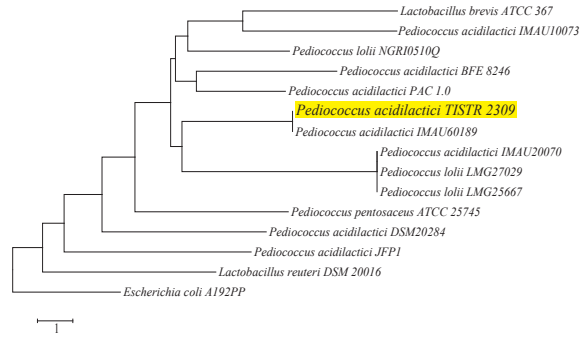

Figure 3: Phylogenetic tree based on 16s rDNA of *P. acidilactici* TISTR 2309 compared with other strains based on the neighbour-joining method. The scale bar represents the number of substitutions per site. The tree was constructed with MEGA v6.0 software.

Data Bank of Japan on a daily basis [24]. Thus the fact that the NCBI and ENA databases gave different results for the percent identities of matching strains is quite surprising. In order to find further relationships between our strain and the strains retrieved from the two databases, the 16S rDNA sequences of the matching species were downloaded from the databases and compared with that of *P. acidlactici* TISTR 2309. For comparison, the nucleotide sequences of *E. coli* A192PP were chosen as outgroup. Furthermore, the sequence of *P. acidlactici* PAC 1.0 which is known to produce pediocin PA-1 was also used for comparison.

The relationship between *P. acidilactici* TISTR 2309 and other strains based on the maximum likelihood method (Figure 2) indicated that this method did not detect any difference between the *P. acidilactici* TISTR 2309 rDNA sequence and the *P. acidilactici* IMAU60189 sequence. *P. acidilactici* TISTR 2309 and *P. acidilactici* PAC 1.0 have the same ancestor but TISTR 2309 is descended by one level and PAC1.0 by two levels from this ancestor. It was also found that TISTR2309 was close to PAC1.0 (number of substitutions 4.2916), but far from *E. coli* A192PP (number of substitutions 54.4267).

When the phylogenetic tree was constructed using the neighbour-joining method (Figure 3), *P. acidilactici* TISTR 2309 was found to be located at the same position as *P. acidilactici* IMAU60189 (number of substitutions 0) but at different positions from *P. acidilactici* PAC 1.0 (8.1448 substitutions) and from *E. coli* A192PP (10.9544 substitutions).

In comparison, the neighbour-joining method is faster in terms of computing than the maximum likelihood method but gives a smaller value for the number of substitution. The neighbour-joining method generates the tree by a hierarchical clustering procedure, whereas the maximum likelihood method finds the most likely genealogical pathway [5]. The results obtained showed that the two methods detected no difference in the rDNA sequences of *P. acidilactici* TISTR 2309 and *P. acidilactici* IMAU60189. They also showed that *P. acidilactici* TISTR 2309 was different but close to *P. acidilactici* PAC 1.0 and that *P. acidilactici* TISTR 2309 was far from *E. coli* A192PP. However there were also obvious differences between the trees constructed by the two methods, particularly for the *Lactobacillus* spp. The maximum likelihood method put the two lactobacilli in the same node, whereas the neighbour-joining method placed them in different nodes.

The phylogenetic trees constructed by the maximum parsimony method (Figure 4) agreed with the results from the previous two methods. Maximum parsimony showed that *P. acidilactici* TISTR 2309 was at the same position as *P. acidilactici* IMAU60189, that *E. coli* was in a distinct outgroup, and that *P. acidilactici* PAC-1.0 was an ancestor of *P. acidilactici* TISTR 2309. Although maximum parsimony displays the detailed phylogram, it does not give the number of substitutions between strains or species. As stated earlier, this cladogram method derives a phylogenetic tree by constructing a tree of shortest length [5]. A comparison of results for *P. acidilactici* TISTR 2309 obtained from the three

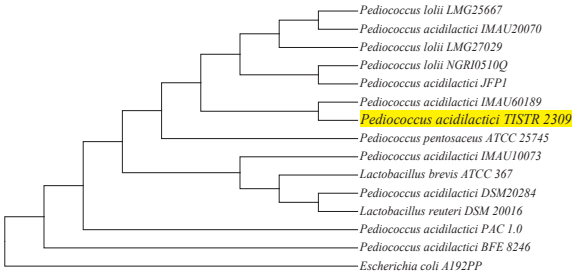

Figure 4: Phylogenetic tree based on 16s rDNA of *P. acidilactici* TISTR 2309 compared with other strains based on the maximum parsimony method. The tree was constructed with MEGA v6.0 software.

cladogram methods with the Blast results from the ENA and NCBI databases showed closer agreement with the ENA results than with the NCBI results.

3.3 *Differentiation of 16S rDNA of P. acidilactici TISTR 2309 during the period of cultivation*

The partial 16S rDNA [9] and full 16S rDNA sequences of *P. acidilactici* TISTR 2309 were compared to find if any differentiation occurred during the 9 years of cultivation. In this study, we used the ClustalX program to identify regions that may indicate functional, structural and/or evolutionary relationships between the two biological sequences. The results showed that differentiation of 2.74% (12/438) occurred in the upper strands of the DNA sequences and of 4.79% (21/438) in the lower strands.

3.4 *Comparison of Bacteriocin Unit (B.U.) of P. acidilactici TISTR 2309 during cultivation period*

Bacteriocinogenic activity was detected when *P. acidilactici* TISTR 2309 (CP7-3) was first isolated [9]. Previous work also showed that TSBYE was suitable for producing bacteriocin as the medium contained no glucose. Therefore acid did not occur during incubation and the pH of the medium remained unchanged [9], [16]. The bacteriocin unit for TISTR 2309 grown in the TSBYE medium was also found to be higher than for that grown in MRS broth [16]. Moreover, when the filtrate was treated with proteinase K, it was found that the bacteriocin unit reduced or disappeared [16]. Therefore the inhibitory activities detected were likely to be produced by a bacteriocinogenic substance. Bacteriocins are defined as peptides that provide bactericidal effect to the bacteria in close proximity to the producer [20]. Therefore a proteinase enzyme could change the structure of the peptides and destroy their bacteriocin property. During the 9 years of cultivation of TISTR 2309, its bacteriocin activity was found to increase significantly from 1.47 ± 0.13 B.U./ml to 3.71 ± 0.17 B.U./ml for inhibition of *B. cereus* and from 1.96 ± 0.01 B.U./ml to 4.16 ± 0.52 B.U./ml for inhibition of *S. aureus* (Table 4).

The increased productivity and performance of bacteriocin units (B.U./ml) found in the present study may be due to different culture conditions. Hongeak [16] cultivated *P. acidilactici* CP7-3 in flask with very low shaking speed (40 rpm), whereas this study was conducted under static conditions in 5% CO_2 incubator and was not completely anaerobic. Dissolved oxygen tension in the broth culture has been reported to influence the level of bacteriocin production by *P. acidilactici* NRRL B5627 [19]. The authors in [19] pointed out that both anaerobic and aeration conditions were unfavorable for pediocin production, while semi-aerobic condition was preferable. The same situation seems to apply to many other antimicrobial peptides produced by lactic acid bacteria. However, many bacteriocins from lactic acid bacteria have been produced under anaerobic conditions because of a general perception of an anaerobic requirement of lactic acid bacteria [15], [19].

In general, the bacteriocin obtained from *Pediococcus* spp. displays primary metabolite kinetics with the rate of production parallel to the growth rate.

Table 4: Comparison of B.U./ml between *P. acidilactici* TISTR 2309 (CP7-3) strains during 9 years of cultivation

Isolated	Tested microorganisms		References
	***B. cereus* (B.U./ml)**	***S. aureus* (B.U./ml)**	
***P. acidilactici* (0 years)**	1.47 ± 0.13	1.96 ± 0.01	[9]
***P. acidilactici* (3 years)**	1.70 ± 0.27	3.36 ± 0.04	[16]
***P. acidilactici* (9 years)**	3.71 ± 0.17	4.16 ± 0.52	This study

The production of bacteriocin via *P. acidilactici* has been reviewed to regulate by the genes on plasmid [15]. If bacteriocin production was plasmid-expressed, then the copy number of plasmids during cultivation could affect the amount of bacteriocin produced.

4 Conclusions

Phylogenetic trees are useful tools for studying the relationship between *P. acidilacti* TISTR 2309 and other species. There are many ways to design and construct a phylogenetic tree. At the present time, the sequences from 16S rDNA are often used in prokaryotic identification due to their appearance in all organisms with an acceptable degree of divergence [5] and because they are included in searchable internet databases. This article has compared the phylogenetic trees constructed by 3 cladogram methods using the full length of the 16S rDNA sequence of *P. acidilactici* TISTR 2309. This work indicates that the species and strains derived from a search of the ENA website for TISTR 2309 provide better identification than from a search of the NCBI website. The MEGA software was used to generate phylogenetic trees by the maximum likelihood, neighbour-joining and maximum parsimony methods. All 3 methods showed that *P. acidilactici* TISTR 2309 was located on the same branch as the strain *P. acidilactici* IMAU60189 which was only found on the ENA database. In contrast the closest strain located in the NCBI database was *P. acidilactici* DSM20284 which was found to have a greater distance from TISTR 2309 in the 3 trees generated by the cladogram methods.

The 16S rDNA sequences of *P. acidilactici* TISTR 2309 were found to change during 9 years of cultivation with the bacteriocin production increasing during this period. We have shown that *P. acidilactici* TISTR 2309 has potential for bacteriocin production. The study of plasmids and their effect on bacteriocin production is of interest for further study.

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