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IDENTIFICATION AND CHARACTERIZATION OF *YERSINIA* FROM FOOD AND ENVIRONMENTAL SOURCES

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ABSTRACT

Yersinia genus includes currently 18 species: *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Yersinia frederiksenii*, *Yersinia intermedia*, *Yersinia kristensenii, Yersinia bercovieri*, *Yersinia mollaretii*, *Yersinia rohdei*, *Yersinia ruckeri*, *Yersinia aldovae*, *Yersinia aleksiciae*, *Yersinia massiliensis*, *Yersinia similis*, *Yersinia entomophaga*, *Yersinia nurmii*, *Yersinia pekkanenii* and *Yersinia wautersii*. The history of the genus *Yersinia* can be dated back to 1883. In 1965, the genus consisted of only three species: *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis.* Since then the taxonomy of the genus has been under tremendous change over the years, and especially the taxonomy of *Y. enterocolitica,* of which many new species have been separated from. Still *Y. enterocolitica* is a group of very heterogeneous bacteria, which can be divided into 6 biotypes and about 30 serotypes and into pathogenic and non-pathogenic strains. This variability makes the identification of *Y. enterocolitica* very challenging.

In the thesis, two *Yersinia* species; *Y. nurmii* and *Y. pekkanenii* are described. These species were characterized by polyphasic taxonomic methods, including of 16S rRNA gene analysis, multilocus sequence analysis (MLSA) of housekeeping genes *glnA, gyrB*, *recA* and *HSP60*, DNA-DNA hybridization studies, 16S and 23S rRNA gene restriction fragment length polymorphism (RFLP), and phenotyping. *Y. nurmii* was isolated from broiler meat packaged under modified atmosphere, and *Y. pekkanenii* from water, soil and lettuce samples. In the 16S rRNA gene analysis and the 16S and 23S rRNA gene RFLP analysis, these two species grouped with other *Yersinia,* but in separate clusters. In the phylogenetic analysis of separate or concatenated housekeeping genes, the species formed unique monophyletic groups in all phylogenetic trees constructed. *Y. nurmii* had a phenotypic profile most similar to that of *Y. ruckeri*. *Y. pekkanenii* could not be differentiated from *Y. pseudotuberculosis* using phenotypic tests.

Methods of polyphasic taxonomy were also used to estimate the taxonomic position of European *Y. enterocolitica* strains of non-pathogenic biotype 1A. *Y. enterocolitica* has been divided into two subspecies; *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica*. Both subspecies consist of pathogenic and non-pathogenic biotypes. In this thesis, 212 *Y. enterocolitica* strains were characterized by numerical analysis of HindIII ribopatterns (16S and 23S rRNA gene RFLP). These strains consisted of 162 strains of biotype 1A and 50 strains of biotypes 2 to 4 isolated from different sources in Europe during years 1997-2013. Phylogenetic relatedness of 20 representative *Y. enterocolitica* strains including 15 biotype 1A strains was further studied by the multilocus sequence analysis of four housekeeping genes (*gln*A, *gyr*B, *rec*A and *HSP60*). The biotype 1A strains studied were found to form a separate genomic group, which differed from *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica*, with suggestion of the existence of a subspecies formed by non-pathogenic *Y. enterocolitica* biotype 1A strains.

Finally, while studying *Enterobacteriaceae* in cold-stored (6 °C), modified atmosphere-packaged (MAP) pig cheek (*musculus masseter*) and hind leg meat (*musculus semimembranosus*), it was noticed that the pathogenic *Y. enterocolitica* subsp. *palearctica* bioserotype 4/O:3 multiplied into high numbers from a non-detectable level in (MAP) pig cheek meat. The *Enterobacteriaceae* isolated in this study were identified by 16S and 23S rRNA gene RFLP using the HindIII enzyme. *Y. enterocolitica* bioserotype 4/O:3 is the most common pathogenic bioserotype word-wide, and it can be transmitted to humans through raw or undercooked pork. Usually the growth of *Enterobacteriaceae* is inhibited by a modified atmosphere with 20% or more CO_2 at refrigerated temperatures. However, in this study, high numbers of *Y. enterocolitica* bioserotype 4/O:3 was observed in MAP cold-stored pig cheek meat, with a concentration of 30% CO_2 and 70% O_2 in the packages.

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I **Murros-Kontiainen, A**.E., Fredriksson-Ahomaa, M., Korkeala, H., Johansson, P. and Björkroth, J. (2011) *Yersinia nurmii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 61(10):2368-2372

II **Murros-Kontiainen, A**.E., Johansson, P., Niskanen, T., Fredriksson-Ahomaa, M., Korkeala H. and Björkroth J. (2011) *Yersinia pekkanenii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 61(10):2363-2367

III **Murros, A.,** Säde, E., Johansson, P., Korkeala, H., Fredriksson-Ahomaa, M. and Björkroth, J. (2016) Characterization of European *Yersinia enterocolitica* 1A strains using restriction fragment length polymorphism (RFLP) and multilocus sequence analysis (MLSA). *Letters in Applied Microbiology* 63(4):282-288

IV Fredriksson-Ahomaa, M., **Murros-Kontiainen, A.,** Säde, E., Puolanne E., and Björkroth, J. (2012) High number of *Yersinia enterocolitica* 4/O:3 in cold-stored modified atmosphere-packed pig cheek meat. *International Journal of Food Microbiology* 155(1-2):69-72

The publications are referred to in the text by their roman numerals. The published articles are reprinted with the permission of their copyright holders.

ABBREVIATIONS

Ail	attachment invasion locus protein
ail	attachment invasion locus
ANI	average nucleotide identity
DDH	DNA-DNA hybridization
dDDH	digital DNA-DNA hybridization
DNA	deoxyribonucleic acid
G+C	guanine/cytosine content of DNA
glnA	glutamine synthetase gene
gyrB	gyrase subunit B gene
HPI	high-pathogenicity island
HSP60	heat shock protein 60 gene
IJSEM	*International Journal of Systematic and Evolutionary Microbiology*
Inv	invasin
inv	invasin gene
MA	modified atmosphere
MAP	modified atmosphere-packaged
MLSA	multilocus sequence analysis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PG	phylogroup
pYV	*Yersinia* virulence plasmid
RFLP	restriction fragment length polymorphism
recA	recombinase A gene
Ribotype	16S and 23S gene restriction fragment length polymorphism type
Ribotyping	16S and 23S gene restriction fragment length polymorphism analysis
RNA	ribonucleic acid
SSC	saline, sodium, citrate
T_m	DNA melting temperature
virF	gene for the trancriptional activator of the *Yersinia* virulence regulon
VRBG	violet red bile agar with glucose
YadA	*Yersinia* adhesion protein
yadA	*Yersinia* adhesion protein gene
YstA	*Yersinia* stable toxin A = enterotoxin A
ystA	*Yersinia* stable toxin A gene
YstB	*Yersinia* stable toxin B = enterotoxin B
ystB	*Yersinia* stable toxin B gene

1 INTRODUCTION

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and it constitutes facultatively anaerobic, oxidase negative and catalase positive bacteria. *Yersinia* cells are Gram-negative straight rods or coccobacilli, carrying DNA with a guanine/cytosine content (the G+C content) of 46-50 mol %. (Kämpfer 2000, Bottone et al. 2015). The genus contains three species which are well recognized human pathogens; *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*.

The first member of the genus, *Y. pestis*, was isolated in 1894 by Alexander Yersin. By 1965, the genus also included *Y. pseudotuberculosis* and *Y. enterocolitica* (Frederiksen 1964, Smith & Thal 1965). Before modern genomic methods were invented, the classification of bacterial species relied on morphologic, cultural and metabolic properties, and the ability to cause disease. Bacterial species are currently characterized by methods of polyphasic taxonomy, which refers to the use of both phenotypic and genotypic data, and phylogenetic information. The taxonomy of *Y. enterocolitica* has changed dramatically over time, and many new species have been separated from it as their own new species. Currently, the genus *Yersinia* includes 18 species.

Yersinia species are typically identified by commercial kits based on biochemical reactions. However, this may be unreliable since there are mostly only small differences between the species. It is extreme difficult to distinguish for example *Y. massiliensis, Y. mollaretii* and *Y. bercovierii* from *Y. enterocolitica*; and *Y. similis, Y. wautersii* and *Y. pekkanenii* from *Y. pseudotuberculosis* (Merhej et al. 2008, Sprague et al. 2008, Murros-Kontiainen et al. 2011b, Savin et al. 2014, Neubauer & Spraque 2015). A biotyping scheme according to Wauters et al. (1987) is widely used for *Y. enterocolitica* to differentiate between pathogenic and non-pathogenic strains. Pathogenic strains belong to biotypes 1B and 2 to 5 (Wauters et al. 1987), and non-pathogenic strains belong to biotype 1A (Bottone 1997). Non-pathogenic strains are widely detected in food and environmental samples (Aleksic & Bockemuhl 1988, Tennant et al. 2003, Fredriksson-Ahomaa 2015). Biotype 5 has only been isolated from hare (McNally et al. 2016). Biotypes 2-4 are low-pathogenic, and are the most common source of human yersiniosis. Strains belonging to biotype 1B are highly pathogenic for humans, however, they have very rarely been found in Europe. (Bottone 1997, Wuthe & Aleksic, 1997, Bottone 1999). The most common pathogenic type found in humans, especially in Europe, is the serotype O:3 belonging to biotype 4. Pigs at slaughter are frequently carrying bioserotype 4/O:3 strains in the tonsils. Contaminated pork is an important source of human yersiniosis in Europe (Tauxe et al. 1987, Ostroff 1995, Fredriksson-Ahomaa et al. 2001, Fredriksson-Ahomaa et al. 2006).

2 REWIEW OF THE LITERATURE

2.1 Bacterial taxonomy

Bacterial species can be described as a group of strains sharing a high degree of similarity in many features tested under highly standardized conditions (Rosselló-Mora & Amann 2001, Stackebrandt et al. 2002). In a strict sense, a prokaryotic species is considered to be a group of strains (including the type strain), which show a DNA-DNA hybridization (DDH) value over 70%, and have less than 5% difference in their DNA melting temperature (ΔT_m) (Stackebrandt et al. 2002).

Polyphasic taxonomy employs a combination of genotypic, phylogenetic and phenotypic information to classify biological entities (Vandamme et al. 1996). Before the development of genotypic and phylogenetic methods, bacterial species were mainly characterized by cultural, morphological, and metabolic features. In the 1957 Bergey's manual of determinative bacteriology (Breed et al. 1957), bacteria were classified by morphological features (such as shape, motility and gram-staining), growth on different media, and biochemical data (use of nutrients and the production of compounds). The habitat and possible pathogenicity were also addressed.

To validly publish a new name for a bacterial species, the new name must be formulated according to the *International Code of Nomenclature of Bacteria* (Lapage et al. 1992, Parker et al. 2015). Nomenclature of bacteria uses two languages; Latin and Greek. The studies describing a new bacterial species are usually published in the *International Journal of Systematic and Evolutionary Microbiology (IJSEM)*. A type strain of the species must be deposited in two culture collections. When the novel species is published in another journal, its name must later be published in a validation list of *IJSEM.*

2.1.1 Taxonomic methods used in this thesis

An ad hoc committee for the re-evaluation of the species concept met in 2002 (Stackebrandt et al. 2002). The committee made various recommendations regarding the definition of species. The ad hoc committee accepted the use of DNA-DNA hybridization (DDH) and difference in DNA melting temperatures (ΔT_m) as a standard method for all new species descriptions. The determination of bacteria to the level of genera using 16S rRNA gene analysis was of particular interest. The committee recommended the use of additional genomic methods, such as multilocus sequence analysis (MLSA) of housekeeping genes, DNA profiling and DNA arrays in species identification. The committee noted that phenotypic characteristics including morphological and physiological characteristics

remained important to recognize the new species, and each species should have a distinct phenotype to identify it from closely related species.

Phenotypic characterization

Krieg and Lockhart (1966) introduced the use of numerical analysis of biochemical characteristics to study the relationships between bacteria. They studied 105 biochemical reactions of 53 strains representing the 12 genera of the family *Enterobacteriaceae*. They were the first to arrange the bacteria into clusters using calculated similarity percentages. Today, commercial kits are available for identification of *Enterobacteriaceae* by phenotypic characteristics, for example the API 20 E kit (bioMérieux) and the Enterotube test (BBL DL Diagnostics) (Smith et al. 1972). False results may arise when using these tests to identify *Yersinia*, however. There are mostly only small differences in biochemical reactions between *Yersinia* species. For example, *Y. massiliensis, Y. mollaretii* and *Y. bercovierii* have the same API 20 E profile as *Y. enterocolitica* (Merheij et al. 2008), and *Y. similis, Y. wautersii* and *Y. pekkanenii* can be misidentified as *Y. pseudotuberculosis* (Sprague et al. 2008, Murros-Kontiainen et al. 2011b, Savin et al. 2014, Neubauer & Spraque 2015). A method by Wauters et al. (1987) is used for biotyping *Y. enterocolitica*.

16S rRNA gene sequencing

Sequencing of genes coding for the small subunit ribosomal 16S rRNA is an important tool for the identification of bacteria based on their phylogenetic relationships. It is one of the primary tests used in the classification of novel species. Stackebrandt and Goebel (1994) introduced a 16S rRNA gene sequence similarity value of 97% to indicate strains belonging to the same species. This threshold value has been suggested to be corrected to 98.7-99.0% (Stackebrandt & Ebers 2006, Kim et al. 2014). However, this method cannot be used alone to differentiate two species. Some species share a value of more than 99% even though they represent different species (Fox et al. 1992).

The DNA-DNA hybridization method

The DNA-DNA hybridization (DDH) method was developed in the 1970s (Ley et al. 1970). This method has remained the standard for delineating bacterial species. It measures the relatedness of whole genome DNA between two bacterial strains. In this method, DNA is first denatured into two complementary DNA strands and is then hybridized into double stranded DNA. Pairing of separate DNA strands depend on the similarity between nucleotide sequences (Ley et al. 1970, Huss et al. 1983). Bacteria which have a relatedness value of 70% or higher, and less than 5% difference in their DNA melting temperature (ΔT_m), are considered to belong to the same species. These values were included in the definition of

bacterial species by the ad hoc committee on reconciliation of approaches to bacterial systematics in 1987 (Wayne et al. 1987).

Multilocus sequence analysis of housekeeping genes

Housekeeping genes can be used for the classification of bacteria and to analyze their evolution (Lawrence et al. 1991). In 2002, the ad hoc committee for the re-evaluation of the species concept recommended the analysis of housekeeping gene sequences when differentiating a species from its neighbors (Stackebrandt et al. 2002). These genes are highly expressed, and highly conserved protein-encoding genes, which are essential for the survival of bacteria. They evolve more slowly than typical protein encoding genes, but more rapidly than rRNA genes (Wertz et al. 2003).

The use of multilocus sequence analysis allows the genotypic characterization of large numbers of isolates or strains and using multiple housekeeping genes. Wertz et al. (2003) introduced a MLSA analysis of five housekeeping genes (*gapA, groEL, gyrA, ompA*, and *pgi*) and the 16S rRNA gene for multiple isolates of species belonging to the genus *Enterobacteriaceae* (*Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Hafnia alvei, Klebsiella oxytoca, Klebsiella pneumoniae*, and *Serratia plymuthica*). Sequence alignments were used to produce phylogenetic trees. They showed that the isolates from each species formed monophyletic groups in the phylogenetic trees constructed, and these were in accordance to the clusters identified by phenotypic data. The authors concluded the existence of genomic clusters, which were in correspondence to traditional species designations.

16S and 23S rRNA gene restriction fragment polymorphism (RFLP) analysis (ribotyping)

The ad hoc committee for the re-evaluation of the species concept in 2002 also recommended the use of additional genetic methods such as DNA profiling and DNA arrays (Stackebrandt et al. 2002). The 16S and 23S rRNA gene restriction fragment length polymorphism (RFLP) analysis, also called ribotyping, is a DNA profiling method used in epidemiological, phylogenetic and taxonomical classification studies. This method was presented in 1986 by Grimont and Grimont (1986) as taxonomic tool. A study by Bouchet et al. (2008) showed that ribotype polymorphisms reflect the polymorphisms of the total DNA, resulting from sequence variability in housekeeping genes typically found to flank rRNA operons.

2.1.2 Whole genome sequence based taxonomic tools

Today, more than 67,000 whole genomes of bacteria have been sequenced, including about 590 *Yersinia* genomes (http://www.ncbi.nlm.nih.gov/genome/browse/). Because of the large amount of available data, methods using whole genome comparisons have been published to replace the traditional wet-lab DDH methods.

Konstantinidis and Tiedje (2005) and Goris et al. (2007) described the use of average nucleotide identity (ANI) and the percentage of conserved DNA as a measurement of genomic relatedness, alternative to DDH. The ANI is based on pairwise genome comparisons of all homologous genomic regions reflecting the degree of evolutionary distance between the genomes. An ANI value of 95-96% corresponds to a DDH value of 70% (Richter & Rosselló-Móra 2009). Varghese et al. (2015) studied 13,151 prokaryotic whole-genomes belonging 3,032 species to investigate the use of ANI in combination with the fraction of orthologous genes (the alignment fraction, AF) to measure the genomic relatedness between two genomes. They proposed a new method for taxonomic species delineation based on AF and ANI.

Auch et al. (2010a) and Meier-Kolthoff et al. (2013a) developed a digital (dDDH) method to determine DDH values *in silico*. They also determined the G+C contents by calculations from the whole-genome sequences. They used the Genome-to-Genome Distance Calculator (GGDC) web server to calculate the values (Auch et al. 2010b). The threshold value for species is also 70%, as is for traditional DNA-DNA hybridization. They also showed that the G+C content, if computed from genome sequences, varies no more than 1% within species. The dDDH values showed better correlation with DDH values than the ANI values. The results were also shown to correlate well with 16S rRNA sequence similarity values, even better than traditional wet-lab DDH values (Meier-Kolthoff et al. 2013b). A threshold value for subspecies has also been suggested to be 79-80% (Meier-Kolthoff et al. 2014). ANI and dDDH values have been used in the description of several novel bacterial species (Nemec et al. 2011, Jiménez et al. 2013, Sawabe 2014, McGinnis et al. 2015, Tong et al. 2015).

2.2 Taxonomy of the genus *Yersinia*

2.2.1 Yersiniae

The lineage of *Yersinia* is:

Domain: *Bacteria*
Phylum: *Proteobacteria*
Class: *Gammaproteobacteria*
Order: *Enterobacteriales*
Family: *Enterobacteriaceae*
Genus: *Yersinia*

The genus *Yersinia* currently includes 18 species; *Y. pestis* (Van Loghem 1944), *Y. pseudotuberculosis* (Smith & Thal 1965), *Y. enterocolitica* (Schleifstein & Coleman 1939, Frederiksen 1964), *Y. ruckeri* (Ewing et al. 1978), *Y. frederiksenii* (Ursing et al. 1980), *Y. intermedia* (Brenner et al. 1980), *Y. kristensenii* (Bercovier et al. 1980b), *Y. rohdei* (Aleksic et al. 1987), *Y. bercovieri* (Wauters et al. 1988), *Y. mollaretii* (Wauters et al. 1988), *Y. aldovae* (Bercovier et al. 1984), *Y. aleksiciae* (Sprague & Neubauer 2005), *Y. massiliensis* (Merhej et al. 2008), *Y similis* (Sprague et al. 2008), *Y. entomophaga* (Hurst et al. 2011), *Y. nurmii* (Murros-Kontiainen et al. 2011a), *Y. pekkanenii* (Murros-Kontiainen et al. 2011b) and *Y. wautersii* (Savin et al. 2014). *Yersinia* has been isolated from many kinds of habitats; human, animals, food and the environment (Bottone et al. 2015).

2.2.2 The history of the genus *Yersinia*

The history of the genus *Yersinia* can be considered to have started in 1883 as Malasez and Vignal reported a disease in guinea pigs. The disease was characterized by causing ovoid nodules in the liver, spleen and lungs, and also swollen mesenteric lymph nodes. This tuberculosis- like disease causing agent was named *Bacillus pseudotuberculosis rodentium* by Pfeiffer in 1889. (Bottone 1992).

In 1939, Scheifstein and Coleman isolated an unidentified group of 5 strains, of which 2 strains were from facial lesions, and 3 strains from feces of human with enteritis. These strains were highly virulent to mice. They were found to be biochemically similar to the bacteria recorded as *Flavobacterium pseudomallei* Whitmore, previously isolated from facial lesions by McIver and Pike in 1934 (Sulakvelidze 2000). Scheifstein and Coleman proposed the name *Bacterium enterocoliticum* for this new, unidentified species (Sulakvelidze 2000).

The type species of the genus is *Y. pestis* (Van Loghem 1944), which is the causative agent of the disease plague (black death), one of the most devastating diseases of human history, which has caused the death of tens of millions people. In 1894, Alexander Yersin isolated this bacterium during a plaque epidemic in Hong Kong (Sulakvelidze 2000). In 1896 Lehman and Neuman described the bacteria, and proposed the name *Bacterium pestis* (cited in Mollaret & Thal 1974). In 1900 the bacteria was placed in the genus *Pasteurella* as *Pasteurella pestis* (cited in Mollaret & Thal 1974). The genus *Yersinia* was first proposed by Van Longhem in 1943 to honour Alexander Yersin. Van Longhem suggested in 1943, that the plague bacillus and "*Bacillus of pseudotuberculosis rodentium"* should be assigned in an own genus, separate in the genus *Pasteurella*. Van Longhem proposed naming them as *Y. pestis* and *Y. rodentium* respectively (Van Loghem 1944). Smith and Thal (1965) agreed with Van Loghem and the two species were placed in the genus *Yersinia* as *Y. pestis* and *Y. pseudotuberculosis.*

Bercovier et al. proposed in 1980 that *Y. pseudotuberculosis* and *Y. pestis* should be classified as two subspecies of *Y. pseudotuberculosis; Y. pseudotuberculosis* subsp. *pseudotuberculosis* and *Y. pseudotuberculosis* subsp. *pestis,* on the bases of DNA-DNA hybridization studies, the same G+C content and similar biochemical reactions (Bercovier et al. 1980c). This reclassification was rejected by the Judicial Commission of the International Committee on Systematic Bacteriology in 1985 because of their radical difference in transmission and pathogenicity.

Y. pestis has been stated to be a clone of *Y. pseudotuberculosis.* (Chain et al. 2004). It has been estimated to evolve from *Y. pseudotuberculosis* 1,500 – 20,000 years ago (Achtman et al. 1999). However, a new study by Reuter et al. (2014) showed that human pathogenic *Yersinia* lineages have evolved independently by ecological separation and acquisition of similar pathogenic determinants, the virulence plasmid and others, as well as functional gene loss and reduced metabolic activity (Reuter et al. 2014). *Y. pestis* has three biovars; Antiqua (a plague pandemic, 541–767 AD), Medievalis (the Black Death and subsequent epidemics from 1346 to the early 19th century), and Orientalis (third pandemic from 1855-1959) (Achtman et al. 1999, Chain et al. 2004).

In 1964, Wilhelm Frederiksen assigned *B. enterocoliticum* to the genus *Yersinia* and changed its name to *Y. enterocolitica* (Frederiksen 1964). Now the genus *Yersinia* consisted of *Y. pestis*, *Y. pseudotubersulosis* and *Y. enterocolitica* (Figure 1).

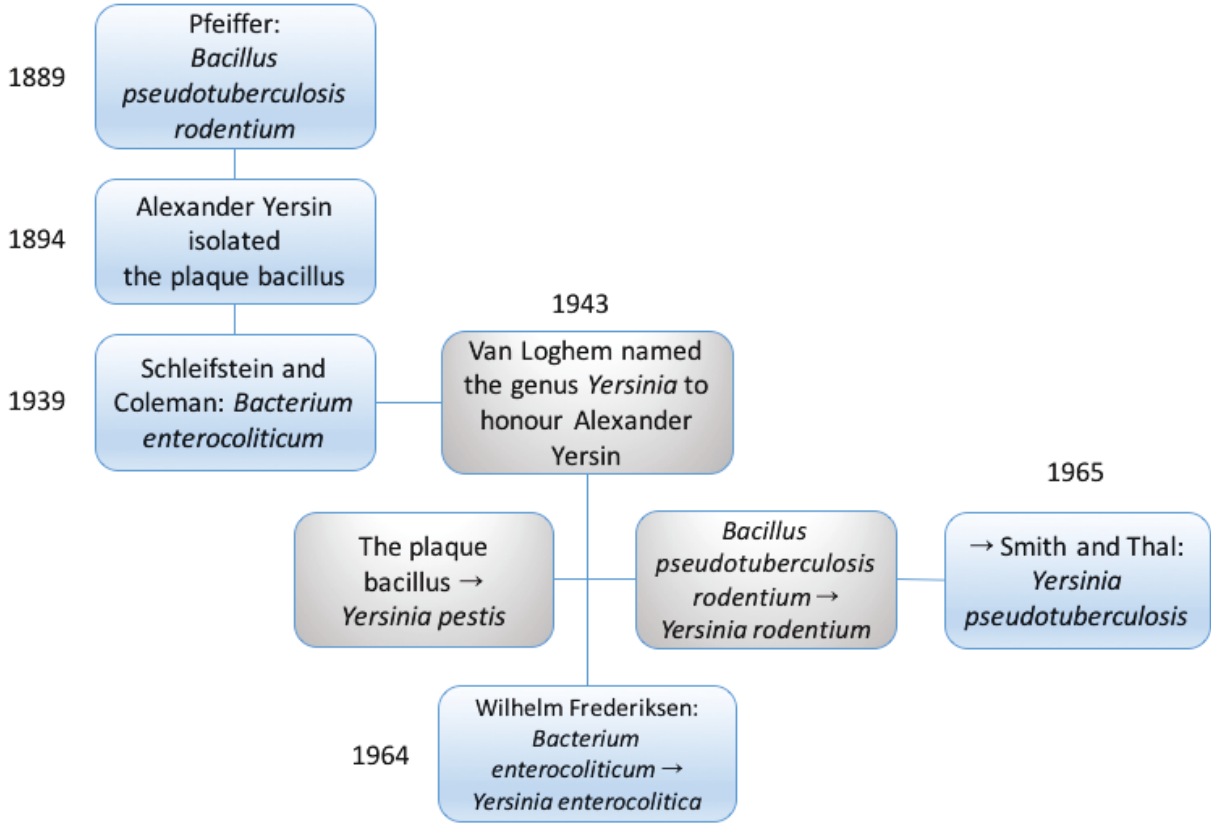

Figure 1. History of the formation of the genus *Yersinia.* Years represent the time the species were reported/described.

2.2.3 The division of *Yersinia enterocolitica* by Wilhelm Frederiksen (1964) into further species

The species *Y. enterocolitica* included many biotypes, and various biotyping schemes were developed based on, for example, the formation of indole, hydrolysis of esculin and fermentation of carbohydrates (Knapp & Thal 1973). The taxonomy of *Y. enterocolitica* became more accurate when Brenner et al. (1976) introduced the use of the DDH technique to study the taxonomy of *Y. enterocolitica* and *Y. pseudotuberculosis* (Brenner et al. 1976).

Many *Yersinia* strains originally identified as *Y. enterocolitica* were later shown to represent different *Yersinia* species (Figure 2). Brenner et al. (1976) found 4 hybridization groups of *Y. enterocolitica*. Further studies demonstrated that these could be divided into 4 distinct species. Bercovier et al. (1980a) presented a detailed phenotypic study to define *Y. enterocolitica sensu stricto*, *Y. intermedia*, *Y. frederiksenii* and *Y. kristensenii* (Bercovier et al. 1980a, Bercovier et al. 1980b, Brenner et al. 1980, Ursing et al. 1980).

A *Y. enterocolitica*- like group, previously designated as group X2 was also recognized as its own species. Bercovier et al. (1984) classified this group as *Y. aldovae,* which was isolated from aquatic ecosystems (drinking water, natural water and fish). *Y. enterocolitica* types 3A and 3B were also given their own species status. They were named *Y. mollaretii* and *Y. bercovieri* (Wauters et al. 1988). These species are difficult to distinguish from *Y. enterocolitica* because they demonstrate very similar biochemical reactions (Merhej et al. 2008), and they share common antigens with *Y. enterocolitica* (Wauters et al. 1988). Aleksic et al. (1987) proposed a new species, *Y. rohdei*, for strains isolated from dog feces, water and stool specimens from humans with diarrhea. The strains had similar biochemical reactions as *Y. enterocolitica* and agglutinated with H antisera of *Y. enterocolitica* but not with *Y. enterocolitica* O antisera. Furthermore, Merhej et al. (2008) isolated two bacterial strains from fresh water in France which had an indistinguishable API 20E biochemical pattern from that of *Y. enterocolitica*. However, using phylogenetic analysis they showed that these strains represent a new species, and the new species was named as *Y. massiliensis*. *Y. frederiksenii* consists of 3 genogroups. *Y. frederiksenii* genogroup 2 has also been suggested to be reclassified as *Y. massiliensis* (Souza et al. 2013).

Y. kristensenii (Bercovier et al. 1980b) consisted of phenotypically heterogeneous strains. Sprague and Neubauer (2005) studied strains of *Y. kristensenii* using 16S rRNA gene sequencing, DNA–DNA hybridization (DDH), determinations of the DNA G+C %, and phenotypic tests. The strains were isolated from human feces, rats, moles, reindeer and pigs, as well as from dairy products word-wide. Based on the results of the tests, a novel species *Y. aleksiciae* was presented (Sprague & Neubauer 2005).

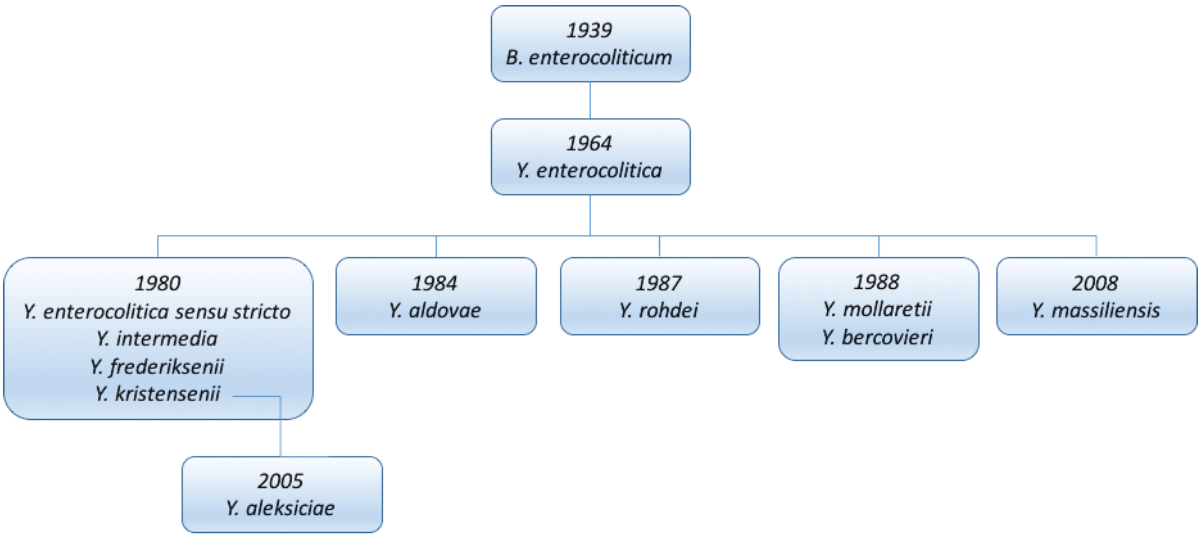

Figure 2. Division of *Yersinia enterocolitica* into different species.

2.2.4 *Yersinia ruckeri, Yersinia entomophaga*, *Yersinia similis* and *Yersinia wautersii*

Y. ruckeri causes a disease called "red mouth disease" for rainbow trout and salmon (Ewing et al. 1978). The disease is a systemic disease, but it is characterized by inflammation of the mouth. Ewing et al. (1978) studied 33 strains isolated from kidney tissue of fish with red mouth disease. They carried out DDH studies of strains of this new species with other species of the family *Enterobacteriaceae*. They were found to be about 30% related to species of both *Serratia* and *Yersinia,* and gave lower values to other members of the family. Biochemically and by their DNA G+C content, the red mouth bacteria were most closely related to *Yersinia,* thus the red mouth bacteria were placed in the genus *Yersinia*.

A bacterial strain identified as a member of a new species *Y. entomophaga* was isolated from diseased larvae of the New Zealand grass grub *Costelytra zealandica* (Hurst et al. 2011). Toxicity of the strain to a variety of insects was studied. The bacteria caused 100% mortality within 2-5 days of ingestion to *Costelytra zealandica* and also to other insects, including the diamondback moth *Plutella xylostella*, the small white butterfly *Pieris rapae*, and the locust *Locusta migratoria*.

Y. similis strains were isolated from guinea-pig, rabbit, mole and mara (Sprague et al. 2008). These strains were initially identified by phenotypic tests as *Y. pseudotuberculosis*. A novel species was confirmed by using 16S rRNA gene sequence analysis, DNA–DNA hybridization, determination of the DNA G+C content, and biochemical tests. Members of *Y. similis* appear to be adapted to the environment, and do not cause disease, but non-pathogenicity for man is thus far unconfirmed.

As described for *Y. similis*, *Y. wautersii* has also been divided from *Y. pseudotuberculosis* (Savin et al. 2014). A group of *Y. pseudotuberculosis* strains designated as the 'Korean group', was placed as a new species based on various analysis including: phenotyping, high resolution electrospray mass spectrometry, 16S rRNA gene sequences, ANI values and MLSA. This publication has been argued by Neubauer and Spraque (2015) as a letter to the editor of the *International Journal of Systematic and Environmental Microbiology*, that strains of *Y. wautersii* should continue to be classified as the 'Korean group' of *Y. pseudotuberculosis*, rather than as a new species because of the lack of adequate evidence in the publication. Savin et al. (2014) used *Burkholderia mallei* and *Burkholderia pseudomallei* as an example of species that should in fact belong to a single species, to explain why their own results were controversial to the rules of the Bacterial code. Neubauer and Spraque (2015) disagreed with this comparison because the classical standard delineation for species are not applied to these two *Burkholderia* species. These have remained named as two different species because they cause distinct medical diseases; *B. mallei* causes glanders in horses, and *B. pseudomallei* melioidosis in humans. This approach has also been applied for *Y. pestis* and *Y. pseudotuberculosis* because of the difference in the diseases they cause, and their very different life cycles.

2.2.5 *Yersinia enterocolitica sensu stricto*

Biotypes of Yersinia enterocolitica

Bercovier at al. (1980a) divided *Y. enterocolitica sensu stricto* into five biotypes based on varied biochemical properties (Table 1). Wauters et al. (1987) added the biotypes to six adding more biochemical reactions (Table 2).

Table 1. Biotypes of *Yersinia enterocolitica sensu stricto* by Bercovier et al. (1980a)

Biochemical reaction	Biotype				
	1	2	3	4	5
Lipase	+	-	-	-	-
Indole	+	+	-	-	-
D-Xylose	+	+	+	-	-
D-Trehalose	+	+	+	+	-
NO reduction	+	+	+	+	-
DNase	-	-	-	+	+
Sucrose	+	+	+	+	-

Table 2. Biotypes of *Yersinia enterocolitica sensu stricto* by Wauters et al. (1987)

Biochemical reaction	Biotype					
	1A	1B	2	3	4	5
Lipase	+	+	-	-	-	-
Indole	+	+	v	-	-	-
Xylose	+	+	+	+	-	v
Trehalose	+	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	-
Salicin (acid 24 h)	+	-	-	-	-	-
Esculin hydrolysis (24 h)	+/-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+/(+)
Voges-Proskauer test	+	+	+	+	+	+/(+)
Pyrazinamidase activity	+	-	-	-	-	-
Sorbose (acid production)	+	+	+	+	+	-

(+) = delayed positive; v = variable

The six biotypes can be divided into three categories based on their pathogenicity. Isolates belonging to biotype 1A are considered to be non-pathogenic (Bottone 1997). Biotype 1B isolates are regarded as highly pathogenic and biotypes 2-5 as moderately pathogenic (Carter 1975). Biotypes 2, 3 and 4 cause most cases of yersiniosis worldwide. Biotype 5 has been isolated from hares (Wuthe & Aleksic 1997).

Somatic (O) antigens

Serologically, *Y. enterocolitica* can be separated into approximately 30 serotypes (Wauters et al. 1991). The O-antigen, which is an outer-membrane lipopolysaccharide, plays an important role in the virulence (Skurnik & Bengoechea 2003). Only a few serotypes are associated with the disease of man and animals (Figure 3). Most common human pathogenic bioserotypes are 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3 and 4/O:3 (Bottone 1999, McNally et al. 2016). Bioserotype 4/O:3 is the most common pathogenic bioserotype worldwide. Bioserotype 2/O:9 also occurs frequently (Ostroff 1995, Bottone 1999). Bioserotype 1B/O:8 occurs mainly in the United States, 2/O:9 in Europe, and 2/O:5,27 occurs world-wide (Ostroff 1995, Bottone 1999). Bioserotype 3/O:3 has been reported in Japan and China (Fukushima et al. 1984, Zheng & Xie 1996). Biotype 1A possesses a vast range of serotypes (Kapperud 1991, Bottone 1999, McNally et al. 2016).

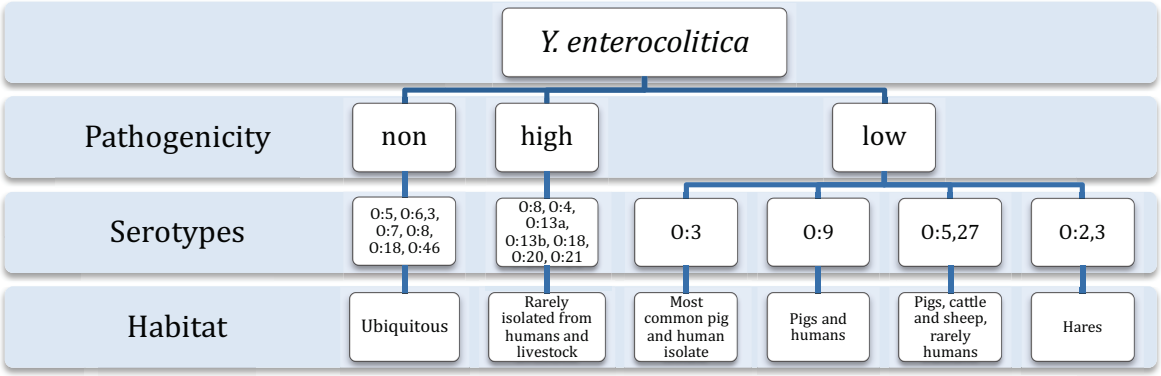

Figure 3. Common serotypes of *Yersinia enterocolitica*, and their usual ecological habitats (Modified from McNally et al. 2016).

Pathogenic Yersinia enterocolitica strains

All pathogenic *Y. enterocolitica* strains carry a 70-kb plasmid, the *Yersinia* virulence plasmid pYV (Gemski et al. 1980). The plasmid has genes for essential virulence factors to prevent phagocytosis, overcome the immune response, and multiply in lymphatic tissues (Cornelis et al. 1998). It includes among others genes for the *Yersinia* outer membrane proteins (Yops) and the *Yersinia* adhesion protein YadA (El Tahir & Skurnik 2001). The highly pathogenic biotype 1B also possess a high-pathogenicity island (HPI) in the chromosome. This is needed to code a yersiniabactin (Ybt) siderophore, which chelates iron, a growth factor for bacteria (Carniel et al. 1996).

Beside the *Yersinia* virulence plasmid, the pathogenic bioserotypes carry chromosomal genes which code for virulence-associated determinants, such as the invasin protein (Inv),

the attachment invasion locus protein (Ail) and the enterotoxin A (YstA) (Miller et al. 1989, Young et al. 1992, Reuter et al. 2014). However, biotype 1A strains have been shown to have chromosomal genes coding for virulence-associated determinants like Ail and enterotoxin B (YstB) (Batzilla et al. 2011, Reuter et al. 2014).

In humans, pathogenic bioserotypes can cause illness, which can range from mild to severe. It has been reported that the infectious dose of the bacteria is about 10^9 organisms. The incubation period of the disease is about 3 to 7 days (Smego et al. 1999). The most common clinical symptoms are diarrhea, low-grade fever, abdominal pain and vomiting. A pseudoappendicitis syndrome, consisting of terminal ileitis and mesenteric lymphadenitis, can occur mostly in children older than 5 years and adults. Septicemia may occur in immunosuppressed patients. Arthritis, myocarditis, glomerulonephritis and erythema nodosum with painful red or purple lesions may occur post infection. (Bottone 1997, Bottone 1999, Zheng et al. 2008).

Non-pathogenic Yersinia enterocolitica strains

Biotype 1A strains are frequently isolated from the environment, food, mammals, insects, reptiles, birds and fish (Aleksic & Bockemuhl 1988, Tennant et al. 2003, Joutsen et al. 2017), and have also been isolated from healthy humans, and sporadically from patients with gastrointestinal symptoms (Tennant et al. 2003, Batzilla et al. 2011, Stephan et al. 2013). These strains are considered non-pathogenic but have been suspected as an opportunistic pathogen that might cause gastrointestinal symptoms in immune-compromised patients, although it lacks the usual virulence factors of the virulent bioserotypes (Batzilla et al. 2011, Bhagat & Virdi 2011). Still, this pathogenic potential is not verified, and requires further investigation (Stephan et al. 2013, Reuter et al. 2014).

2.2.6 Taxonomic division of *Yersinia enterocolitica* into subspecies

Subspecies is the lowest taxonomic category that has official standing in nomenclature. Subspecies is defined as "genetically close organisms, which differs in selected attributes such as phenotype or genetic properties, but represent the same species by their DNA-DNA hybridization values" (Wayne et al. 1987).

Y. enterocolitica has been divided into two subspecies; *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica* (Neubauer et al. 2000) . The division was based on differences in the sequences of the 16S rRNA genes, and DNA-DNA hybridization studies. Neubauer et al. (2000) demonstrated specific 16S rRNA gene sequences, which distinguished the subspecies from each other. *Y. enterocolitica* subsp. *enterocolitica* was characterized by the partial sequence 5'-

AAGGCCAATAACTTAATAGCTTGTTGGATT-3' (451-480 nt of M59292) and *Y. enterocolitica* subsp. *palearctica* by the partial sequence 5'-AAGGCATAAAGGTTAATAACCTTTGTGATT-3' (451-480 nt of U63135). The type strain of *Y. enterocolitica* ATCC 9610 (bioserotype 1A/O:7,8) was assigned as the reference strain of *Y. enterocolitica* subsp. *enterocolitica,* and DSMZ 13030 (bioserotype 4/O:3) as the reference strain of *Y. enterocolitica* subsp. *palearctica*. In the study, strains of bioserotypes 1B/O:8, 1B/O:13, 1B/O:18, 1B/O:20 and 1B/O:21 displayed the same 16S rRNA sequence as the type strain *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610 (bioserotype 1A/O:7,8). Strains of bioserotypes 1A/O:5, 1A/O:6,30, 1A/O:7,8, 2/O:9 and 3/O:5,27 displayed the same 16S rRNA sequence as *Y. enterocolitica* subsp. *palearctica* DSMZ 13030 (bioserotype 4/O:3) (Table 3.).

Table 3. Division of bioserotypes of the two *Yersinia enterocolitica* subspecies by Neubauer et al. (2000)

Subspecies	Bioserotype	Pathogenicity	Type strain
***Y. enterocolitica* subsp. *enterocolitica*:**	1A/O:7,8	-	ATCC 9610
	1B/O:8	+	
	1B/O:13	+	
	1B/O:18	+	
	1B/O:20	+	
	1B/O:21	+	
***Y. enterocolitica* subsp. *palearctica*:**	1A/O:5	-	
	1A/O:6,30	-	
	1A/O:7,8	-	
	2/O:9	+	
	3/O:5,27	+	
	4/O:3	+	DSMZ 13030

Y. enterocolitica subsp. *palearctica* DSMZ 13030 gene type was noticed to separate into two clusters when studied by DNA-DNA hybridization. One consisted of non-pathogenic bioserotypes (1A/O:5, 1A/O:6,30, 1A/O:7,8), and the other by the pathogenic bioserotypes (2/O:9, 3/O:5,27 and 4/O:3). The DDH values in these groups was high, more than 92%. Both subspecies consist of both non-pathogenic and pathogenic bioserotypes. The type strain of *Y. enterocolitica* subsp. *enterocolitica* is non-pathogenic compared to the other strains of the subspecies, which are pathogenic.

Rakin et al. (2012) studied differences in virulence factors of *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica* using whole genome sequencing. The phylogeny of these subspecies was also studied. The results clearly indicated the existence of three gene clusters representing three different *Y. enterocolitica* groups. Biotype 1A strains exhibited a large genetic distance from both *Y. enterocolitica* subspecies.

Howard et al. (2006) studied 94 strains of *Y. enterocolitica*, consisting of 35 human, 35 pig, 15 sheep and 9 cattle isolates from non-pathogenic, low-pathogenic and highly pathogenic biotypes using whole-genome comparisons with DNA microarrays. Analysis revealed three distinct clusters composed of non-pathogenic strains, low-pathogenic strains, and a highly pathogenic strains. Analysis of the core genes supported the high heterogenicity of the species. These findings indicated to supported the existence of three subspecies of *Y. enterocolitica*.

To study the phylogenetic relationship between high, low, and non-pathogenic *Y. enterocolitica* strains, Reuter et al. (2012) sequenced the whole genome of each of the *Y. enterocolitica* biotypes. In the resulting phylogenetic tree (Figure 4), the non-pathogenic 1A and high-pathogenic 1B biotypes were more closely related to each other than to the low-pathogenic biotypes.

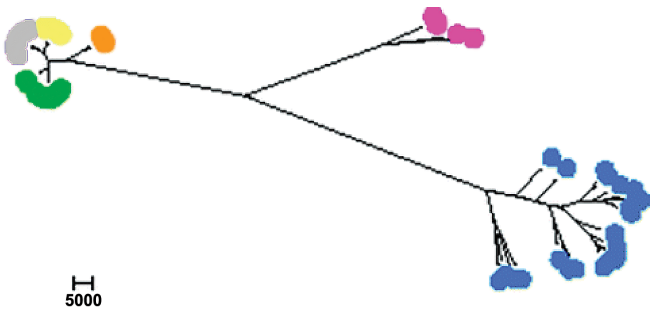

Figure 4. Phylogeny of different *Yersinia enterocolitica* biotypes studied by maximum-likelihood analysis. Biotype 1A strains are indicated by *blue circles*, biotype 1B strains by *purple circles*, biotype 5 strains by *orange circles*, bioserotype 4/O:3 strains by *gray circles*, bioserotype 3/O:5,27 strains by *yellow circles*, and biotype 2 and bioserotype 3/O:9 strains by *green circles* (Reuter et al. 2012). Published with a license from Springer and Copyright Clearance Center.

Reuter et al. (2012) also sequenced housekeeping genes previously used for MLSA studies in *Y. pestis* and *Y. pseudotuberculosis* (Achtman et al. 1999, Bottone 1999) for over 100 *Yersinia* strains representing all current species of the genus *Yersinia*. In the resulting phylogenetic tree (Figure 5), human pathogenic *Y. enterocolitica*, *Y. pestis/Y. pseudotuberculosis*, *Y. ruckeri* and the studied environmental species formed separate complexes. Also in this phylogenetic tree in the *Y. enterocolitica* complex, non-pathogenic, high-pathogenic and low-pathogenic biotypes form separate clusters.

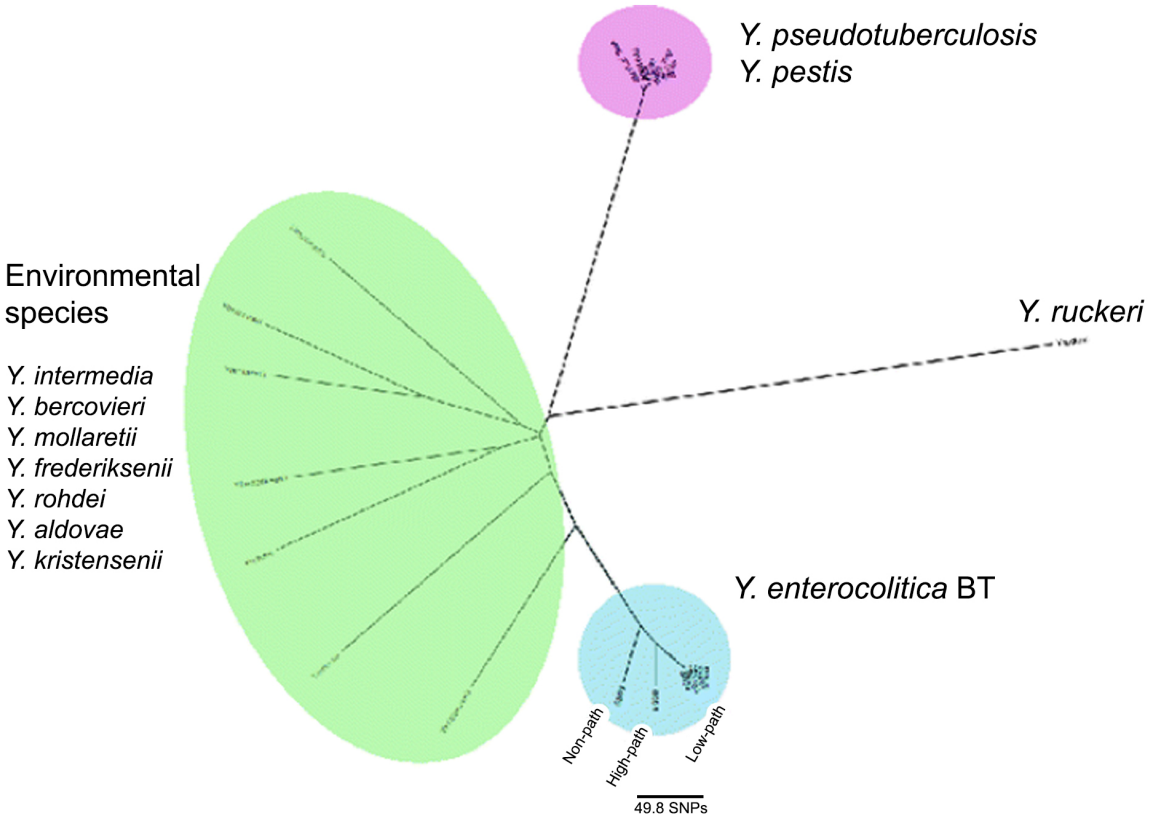

Figure 5. Maximum likelihood phylogeny showing the phylogenetic analysis of the *Yersinia* genus (Reuter et al. 2012). Published with a license from Springer and Copyright Clearance Center.

3 AIMS OF THE STUDY

The aim of this study was to identify and characterize *Yersinia* isolates recovered from different sources using methods of polyphasic taxonomy.

The specific aims of the individual studies were:

I to study whether strains isolated from broiler meat packaged under a modified atmosphere represent a new species of the genus *Yersinia,*

II to study whether strains isolated from water, soil and lettuce samples represent a new species of the genus *Yersinia,*

III to estimate the taxonomic position of European non-pathogenic *Y. enterocolitica* strains of biotype 1A

IV to identify and to study the numbers of *Enterobacteriaceae* in modified atmosphere packaged pig cheek meat (*musculus masseter*) and hind leg meat (*musculus semimembranosus*) during cold storage at 6°C, using 16S and 23S gene RFLP analysis,

V to assess the suitability of 16S and 23S gene RFLP analysis, multilocus sequence analysis of housekeeping genes *glnA, gyrB*, *recA* and *HSP60*, 16S rRNA gene analysis and phenotyping, for the identification of *Yersinia* species.

4 MATERIALS AND METHODS

4.1 Bacterial isolates (I-IV)

Study I

Three unknown enterobacterial isolates were studied. These originated from three packages of broiler meat cuts, packaged under a modified atmosphere. They were recovered from VRBG (violet red bile agar with glucose) medium used for the isolation and enumeration of enterobacteria in the products. In the analysis of the HindIII and EcoRI ribotype patterns of 16S and 23S rRNA genes, the strains were preliminarily identified as members of the family *Enterobacteriaceae,* but the patterns did not cluster together with those of any known species.

Study II

Three strains isolated from water, lettuce and soil samples were studied. These strains originated from the study of Niskanen et al. (2009), in which they had been preliminarily identified as *Y. pseudotuberculosis* by phenotypic tests, but they lacked virulence encoding genes *inv* and *virF*. Information obtained from NotI digests using pulsed-field gel electrophoresis (PFGE), HindIII and EcoRI ribotype patterns and the O-antigene gene clusters were also not in accordance with the phenotypic test results.

Study III

In this study, 212 *Y. enterocolitica* strains, the *Y. enterocolitica* subsp. *enterocolitica* type strains ATCC 9610^T, the *Y. enterocolitica* subsp. *palearctica* type strain DSM 13030^T, and nine reference strains obtained from the study of Neubauer et al. (2000) were characterized by 16S and 23S rRNA gene RFLP using the HindIII enzyme. The 212 strains had been isolated from human, animal and food samples in Europe during 1997-2013. These included 162 non-pathogenic biotype 1A strains and 50 strains of biotypes 2, 3 and 4. Twenty representative strains were selected for MLSA analysis. These included 15 biotype 1A strains, one strain of biotype 2, one strain of biotype 4, one reference strain of biotype 1B and the two type strains (biotype 1A and 4).

Study IV

The growth of psychrotrophic enterobacteria was studied in cold-stored MAP pig cheek meat (*musculus masseter*) and hind leg meat (*musculus semimembranosus*). Ribotyping of 16S and 23S rRNA genes with the HindIII restriction enzyme was used to identify bacterial strains using the department's large ribopattern database. Forty samples per muscle type

were packaged under a modified atmosphere of 30% CO_2/ 70% O_2 and stored 12 days at 6°C. Twenty samples per muscle type were studied on day 1 and on day 13. In total, 170 oxidase-negative isolates from VRBG were studied.

4.2 DNA extraction (I-IV)

DNA for analysis was extracted according to the method of Pitcher et al. (1989) with the addition of 0.6 mg proteinase K to the cell suspension buffer.

4.3 16S and 23S rRNA gene RFLP analysis (I-IV)

Ribotyping procedures were performed as described by Niskanen et al. (2009). DNA was cleaved with HindIII and EcoRI restriction endonucleases in studies I and II and with HindIII restriction endonuclease in studies III and IV. The DNA fragments were separated by agarose gel electrophoresis. Southern blotting was used to transfer the fingerprint patterns to a nylon membrane with a vacuum-blotting device (Vagugene, Pharmacia, Uppsala Sweden). DNA fragments were fixed to the nylon membrane by UV light. Five probes targeting the 16S and 23S rRNA-encoding genes were labeled as described by Blumberg et al. (1991) during the reverse transcription reaction. Membranes were hybridized at 58°C overnight and digoxigenin-labelled fragments (ribopatterns) were detected as recommended by Roche Molecular Biochemicals.

The ribopatterns were scanned, converted to tagged image file format images, and analyzed by BioNumerics 5.10 software (Applied Maths). Ribopatterns were normalized based on DNA molecular weight markers. Dice coefficients were used for estimating similarity between patterns. The non-weighted pair-group method was used with an arithmetic mean clustering algorithm (UPGMA) for the construction of dendrograms.

4.4 The *Enterobacteriaceae* ribopattern database (I-IV)

A database of enterobacteria of the Department of Food Hygiene and Environmental Health, University of Helsinki contains HindIII ribopatterns of over 2000 psychrotrophic *Enterobacteriaceae* strains. The database also contains type and reference strains of food associated enterobacteria. It utilizes 16S and 23S rRNA gene HindIII ribopatterns as operational taxonomic units in numerical analysis.

4.5 Sequencing of the 16S rRNA, *glnA, gyrB, rec*A and *HSP60* genes (I-III)

Kotetishvili et al. (2005) studied the genetic relationships of 58 *Yersinia* strains using multilocus sequence analysis of the 16S rRNA gene, and four housekeeping genes; *glnA* (encoding glutamine synthetase), *gyrB* (DNA gyrase subunit B), *recA* (recombinase A) and *HSP60* (heat shock protein 60). This method was used in studies I-III (Kotetishvili et al. 2005). This MLSA method suited well for housekeeping genes *glnA, recA* and *HSP60*. For the *gyrB* gene, and the 16S rRNA gene, PCR-conditions were modified. The *glnA, gyrB, recA* and *HSP60* genes were amplified using primers described by Kotetishvili et al. (2005) (Table 4). Primers were custom made by Oligomer Oy, Helsinki, Finland.

Table 4. Primers used in the multilocus sequence analysis

Gene	Primers (5′→3′)	Fragment
16S rRNA	CTGGCTCAGGAYGAACGCTG AAGGAGGTGATCCAGCCGCA	1,425 bp
glnA	CGATTGGTGGCTGGAAAGGC TTGGTCATRGTRTTGAAGCG	494 bp
gyrB	CGGCGGTTTGCAYGGYGTRGG CAGSGTRCGRGTCATYGCCG	471 bp
recA	GGGCCAAATTGAAAARCARTTCGG CGCCRATYTTCATRCGRATYTGGTY	483 bp
HSP60	GACGTNGTAGAAGGTATGYAG CGCCGCCAGCCAGTTTAGC	486 bp

The PCR cycles for amplification of the *HSP60*, *glnA* and *recA* genes were as follows: denaturation at 94°C for 5 min, followed by 30 amplification cycles, (94°C for 45 s, 51°C for 45 s and 72°C for 1 min), and the final elongation at 72°C for 5 min. For *gyrB*, the annealing temperature had to be increased to 62°C to obtain PCR products.

Universal primers F19-38 and R154-1522 were employed, instead of those described in the study of Kotetishvili et al. (2005), yielding 16S rRNA gene fragments of 1452 bp (Table 4). The PCR cycles were as follows: denaturation at 98°C for 2 min, 30 amplification cycles (93°C for 1 min, 54°C for 2 min and 72°C for 2.5 min) and final elongation at 72°C for 8 min.

The PCR products were sequenced in both directions using Applied Biosystems BigDye Terminator v.3.1 Cycle Sequencing kit and an ABI 3130XL Genetic analyzer at the Institute if Biotechnology, University of Helsinki.

The consensus sequences were analyzed using BioNumerics 5.10 software. The sequences were aligned with 16S rRNA and housekeeping genes *glnA, gyrB, recA* and *HSP60*, of type and reference strains of all current *Yersinia* species.

4.6 DNA G+C content analysis and DNA-DNA hybridization studies (I-II)

The DNA G+C content of the isolates was determined by the Light Cycler instrument as described by Xu et al. (2000). To minimize experimental errors, a reference DNA was used as a standard, and the G+C content was calculated as reported by mol% G+Cx = mol% (G+C)r+1.4652(Tmx-Tmr)+0.0063(Tmx^2-Tmr^2), where Tm is the thermal melting temperature, x is the unknown organism and r is the reference organism. Sharp melting curves were achieved by using 1 x SSC. The DDH tests were performed with an optical method (Ley et al. 1970) at the DSMZ Braunschweig, Germany.

4.7 Morphology and phenotypic tests (I-IV)

To determine the morphological properties, the isolates were examined by Gram-staining. Transmission electron microscopy was used to determine the morphological properties of type strains representing new species in studies I-II. This was done with a Jeol 1200 EX II electron microscope (Jeol Ltd, Tokyo, Japan) at the Electron microscopy unit of the Institute of Biotechnology, University of Helsinki. Biochemical characterization of *Yersinia* strains in studies I and II were determined with API 20 E and API 50 CH strips according to manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, France). The incubation temperature used was 28°C and results were read after 24, 48 and 72 h. In studies III and IV, the biochemical characteristics were determined using API 20E tests (bioMérieux SA, Marcy l'Etoile, France) with the incubation time of 18-20 h at 25°C. In addition, biotyping using pyrazinamidase and tween activity, esculin hydrolysis, indole production, and xylose, salicin and trehalose fermentation tests as described by Wauters *et al*. (1987), was included in all studies (I-IV). Oxidase activity was tested with oxidase strips and catalase activity with 3% hydrogen peroxide. Motility testing in studies I and II was performed at 28 and 37°C with Motility Test and Maintenance and Motility Test media (Atlas & Snyder, 2006). Each biochemical test was done at least twice.

4.8 Determination of virulence genes (I-IV)

In studies I, II and IV, the presence of virulence genes *virF*, *inv*, *ail* and *yadA* were determined as described by Fredriksson-Ahomaa et al. (1999) and Niskanen et al. (2009). In study III, the presence of virulence genes *virF*, *ail*, *ystA* and *ystB* were studied by real-time PCR method as described by Joutsen et al. (2013).

5 RESULTS AND DISCUSSION

5.1 Study I: *Yersinia nurmii* sp. nov

Three unknown isolates APN5b-c, ABF6a-c and APN3a-c ^{T} from MAP broiler meat, were studied using a polyphasic approach. This included multilocus sequence analysis of genes *glnA*, *gyrB*, *recA*, *HSP60* and 16S rRNA, numerical analyses of HindIII and EcoRI ribopatterns, DDH reactions with the closest phylogenetic neighbors and phenotyping.

Strains APN5b-c, ABF6a-c and APN3a-c ^{T} shared 97.8–98.7% 16S rRNA gene sequence similarity with other *Yersinia* species. Phylogenetic analyses of genes *glnA*, *gyrB*, *recA*, *HSP60* and 16S rRNA were performed by both the neighbor-joining and maximum-parsimony methods. The strains formed a monophyletic group that was separate from other *Yersinia* species in all phylogenetic trees constructed, either from individual genes or concatenated *glnA*, *gyrB*, *recA* and *HSP60* gene sequences. In the trees constructed from *glnA*, *recA* and *HSP60* gene sequences, *Y. ruckeri* was positioned as the closest phylogenetic neighbor. The phenotypic profile of strains APN5b-c, ABF6a-c and APN3a-c ^{T} was most similar to that of *Y. ruckeri*. In the numerical analysis of both HindIII and EcoRI ribopatterns, strains APN5b-c, ABF6a-c and APN3a-c ^{T} formed a unique cluster.

DDH reactions were performed with APN3a-c^{T} and ABF6a-c against the type strains *Y. ruckeri* ATCC 29473 ^{T}, *Y. rohdei* DSM 18270 ^{T} and *Y. frederiksenii* DSM 18490 ^{T}. *Y rohdei* DSM 18270 ^{T} was chosen because it was phylogenetically close to *Y. nurmii* in 16S rRNA gene sequence analysis performed by both neighbor-joining and maximum-parsimony methods. The DDH values against these species varied from 17.5 to 33.4%, and the value between strains APN3a-c^{T} and ABF6a-c was 81.7%. Based on the data, a novel species, *Yersinia nurmii* was proposed.

Y. nurmii has resemblance with *Y. entomophaga* (Hurst et al. 2011), which was published in the same issue of *International Journal of Systematic and Evolutionary Microbiology* in 2011. In multilocus sequence analysis of genes *dnaJ, glnA, gyrB, groEL* and *recA*, the *Y. entomophaga* was most closely related to *Y. ruckeri*. In addition, *Y. ruckeri* was the closest phylogenetic neighbor of *Y. nurmii* in our publication (I). In the study of Reuter et al. (2014), *Y. nurmii* and *Y. entomophaga* were closest phylogenetic neighbors to each other, and were in the same branch with *Y. ruckeri* in the phylogenetic trees (Figure 6). Some metabolic pathways were found to be absent from a distinct branch of the phylogenetic tree occupying *Y. pestis, Y. pseudotuberculosis, Y. similis, Y. ruckeri, Y. nurmii* and *Y. entomophaga*. The writers concluded from the data that this would likely be due to early ecological differentiation.

The phenotypic profiles of *Y. nurmii* and *Y. entomophaga* are both most similar to *Y. ruckeri*; and when compared to each other, the profiles are very similar. Few biochemical reactions differed. In contrast to other *Yersinia* species, *Y. ruckeri, Y. nurmii* and *Y. entomophaga* do not hydrolyse urea, and do not ferment L-arabinose.

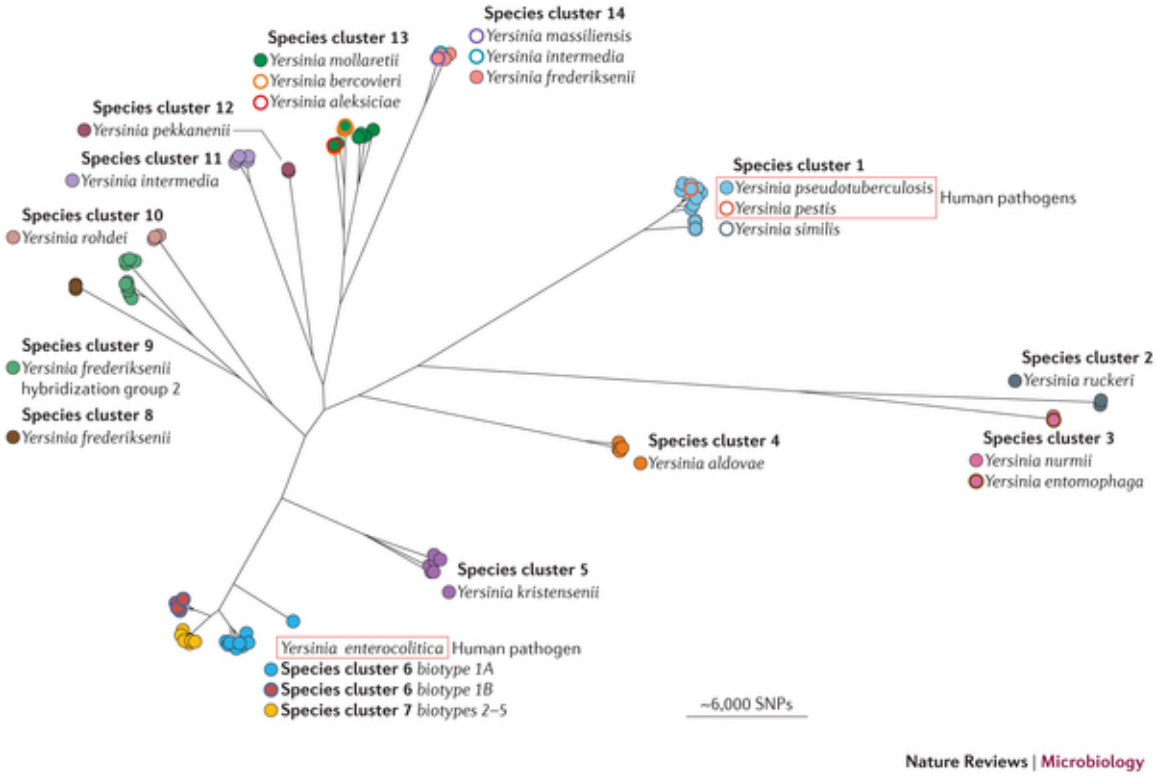

Figure 6. Phylogeny of the genus *Yersinia* based on maximum-parsimony analysis of 84 housekeeping genes (From McNally et al. 2016). Published with a license from Springer and Copyright Clearance Center.

Y. entomophaga is toxic to a variety of insects, and was isolated from diseased larvae of the New Zealand grass grub, *Costelytra zealandica* (Hurst et al. 2011). *Y. ruckeri* causes enteric red mouth disease to salmonid fish (Ewing et al. 1978). Reuter et al. (2014) studied 41 pathogenicity determinants of 94 strains representing all *Yersinia* species. The distribution of the genes fell into three categories: "genes represented in all lineages of the genus, genes gained or lost by entire lineages, and genes represented in only single isolates". All of the studied pathogenicity determinants were the same in *Y. nurmii* and *Y. entomophaga*. This indicates that *Y. nurmii* could also be pathogenic to insects, but this has not been studied. It is evident that *Y. nurmii* and *Y. entomophaga* are very closely related. However, a dDDH value of 59.3% between *Y. nurmii* and *Y. entomophaga* [calculated using the genome-to-genome distance calculator (GGDC 2.0) http://ggdc.dsmz.de/distcalc2.php according to

Meier-Kolthoff et al. (2013)], and a ANI value of 94.80% [calculated using EzGenome (http://www.ezbiocloud.net/ezgenome/ani), which uses the ANI algorithm described as Goris et al. (2007)], support the finding that they represent unique species (personal information from Dr Per Johansson). Between *Y. nurmii* and *Y.ruckeri* a dDDH value of 25.30% and an ANI value of 81.97% can be calculated (personal information from Dr Per Johansson). Whole genome sequences for these calculations can be obtained from the database of the National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/genome/browse/.

As it has been debated if *Y. ruckeri* is actually a member of the genus *Yersinia*. The identification of *Y. nurmii* as well as *Y. entomophaga* support the position of *Y. ruckeri* in the genus *Yersinia* (Figure 6.).

5.2 Study II: *Yersinia pekkanenii* sp. nov

Strains Å125KOH2, ÅYV25K1 and ÅYV7.KOH2^T originated from water, soil and lettuce samples and had been identified as *Y. pseudotuberculosis* by phenotypic tests in a previous study by Niskanen et al. (2009). Their API 20E identification was 98.4% *Yersinia pseudotuberculosis*. The O-antigen gene clusters were different to that of *Y. pseudotuberculosis,* and the strains also lacked virulence encoding genes *inv* and *virF*. Thus, the strains were subjected to further studies.

In the numerical analysis of both HindIII and EcoRI ribopatterns, and in the phylogenetic analysis of genes *glnA, gyrB*, *recA*, *HSP60* and 16S rRNA performed by both the neighbor-joining, or maximum-parsimony methods, the strains Å125KOH2, ÅYV25K1 and ÅYV7.KOH2^T formed a monophyletic group that was separate from other *Yersinia* species in all phylogenetic trees constructed either from individual genes, or concatenated *glnA, gyrB*, *recA* and *HSP60* sequences. In analysis of the 16S rRNA gene sequences of Å125KOH2, ÅYV25K1 and ÅYV7.KOH2^T, the strains were phylogenetically most closely related to *Y. aldovae* and *Y. mollaretii*, but DDH values between these species showed that the strains represented an own species. They also had a unique biochemical profile. Based on the data, *Yersinia pekkanenii* was proposed.

ANI values between *Y. pekkanenii* and other members of the genus vary from 77.28% to 85.16% and dDDH values from 21.90% to 29.20% (personal information from Dr Per Johansson). Also based on these results, *Y. pekkanenii* represent a novel species in the genus.

The study of *Y. pekkanenii* shows that the identification of *Y. pseudotuberculosis* can lead to incorrect results if the identification is only based by biochemical reactions. In the study of Niskanen et al. (2009), the strains could not be serotyped and the typical virulence genes (*virF* and *inv*) were missing. In such cases, the strains may represent *Y. pekkanenii*.

5.3 Study III: Taxonomic position of European non-pathogenic *Yersinia enterocolitica* biotype 1A strains

The 16S and 23S rRNA gene RFLP analysis using HindIII restriction enzyme showed three main ribogroups among 223 *Y. enterocolitica* strains studied. All 162 European non-pathogenic biotype 1A strains, and the three biotype 1A strains obtained from Neubauer et al. (2000) belonged to a single ribogroup. Highly pathogenic biotype 1B strains formed a distinct ribogroup. These included the type strain ATCC 9610^T of Y. *enterocolitica* subsp. *enterocolitica*, and three biotype 1B reference strains. The third ribogroup was formed from strains belonging to biotypes 2 to 4. It included the 50 studied pathogenic strains of biogroups 2-4, the type strain DSM 13030^T of *Y. enterocolitica* subsp. *palearctica* (biotype 4) and reference strains of biotypes 2-4 obtained from Neubauer et al. (2000). Fifteen biotype 1A strains isolated from different sources from Finland, Germany and Switzerland were selected for MLSA analysis of housekeeping genes *glnA, gyrB, recA* and *HSP60*, and ten strains for the analysis of the 16S rRNA gene. These strains carried the *ystB* genes and four of these also the *ail* gene. Genes *ystA* and *virF* were not detected.

Phylogenetic analysis of individual or concatenated neighbor-joining and maximum-parsimony housekeeping genes showed that the 15 European *Y. enterocolitica* 1A strains formed a separate phylogenetic group. Phylogenetic analysis of the 16S rRNA gene grouped the ten selected European *Y. enterocolitica* 1A strains together with the type strain DSM 13030^T of *Y. enterocolitica* subsp. *palearctica* biotype 4.

The results from the 16S and 23S rRNA gene RFLP analysis and phylogenetic analyses of housekeeping genes *glnA, gyrB, recA* and *HSP60* showed that the non-pathogenic European *Y. enterocolitica* 1A strains formed a genomic group which was distinct from *Y. enterocolitica* subsp. *palearctica* and *Y. enterocolitica* subsp. *enterocolitica*. This indicates that European *Y. enterocolitica* biotype 1A strains may form an own subspecies.

Based on the variation of the 16S rRNA sequences, Neubauer et al. (2002) divided *Y. enterocolitica* into two subspecies; subsp. *enterocolitica* and subsp. *palearctica*. Nonetheless, 16S rRNA gene analysis has been recommended to be used in the identification of bacteria only to the level of genera (Stackebrandt et al. 2002). It is unreliable to use this gene for species or subspecies level differentiation since it lacks enough variation.

In order to study the evolution of pathogenic *Yersinia* Reuter et al. (2014) published a study of whole genome sequencing of 241 strains representing all current *Yersinia* species. Analysis of the concatenated sequences of 84 housekeeping genes and the virulence plasmid pYV indicated that early ecological separation had split the human pathogenic *Yersinia* strains from the environmental *Yersinia* species and non-pathogenic *Y. enterocolitica* 1A biotypes. During evolution, human pathogenic *Yersinia* then acquired similar pathogenicity determinants such as the virulence plasmid pYV and the attachment invasion locus *ail*, but

became more metabolically restricted. Pathogenic *Y. enterocolitica* biotype 1B and biotypes 2-5 were found to have had more gene loss than non-pathogenic biotype 1A. Analysis of 380 metabolic tests showed environmental 1A strains to have a much broader metabolic activity than strains of biotypes 1B and 2-5.

Reuter et al. (2014) divided *Y. enterocolitica* into six phylogroups (PGs) using whole genome analysis and core genome single nucleotide polymorphism (SNP) based phylogenetic analysis of 94 strains of *Y. enterocolitica*. The non-pathogenic biotype 1A and the highly pathogenic biotype 1B formed discrete clusters (PG1 and PG2 respectively) in the resulting phylogenetic trees, whereas the low-pathogenic biotypes 2–5 consisted of four closely related lineages (PGs 3-6). Phylogroups 3-6 formed tight clusters compared with phylogroups 1 and 2. This revealed low genetic diversity within phylogroups 3–6. The non-pathogenic phylogroup 1 (biotype 1A) has found to be most similar to ancestral *Y. enterocolitica* (McNally et al. 2016).

Using the data from Reuter et al. (2014), Hall et al. (2015) continued the work to develop a seven gene MLSA scheme that would rapidly and accurately identify any member of the *Yersinia* genus. The results were in concordance with the whole-genome phylogeny of the species (Reuter et al. 2014). Also in this study, biotype 1A (PG1) and biotype 1B (PG2) represented own clusters. Low-pathogenic biotypes 2-5 represented a cluster, which could be further subtyped on the basis of serotypes. (Figure 7).

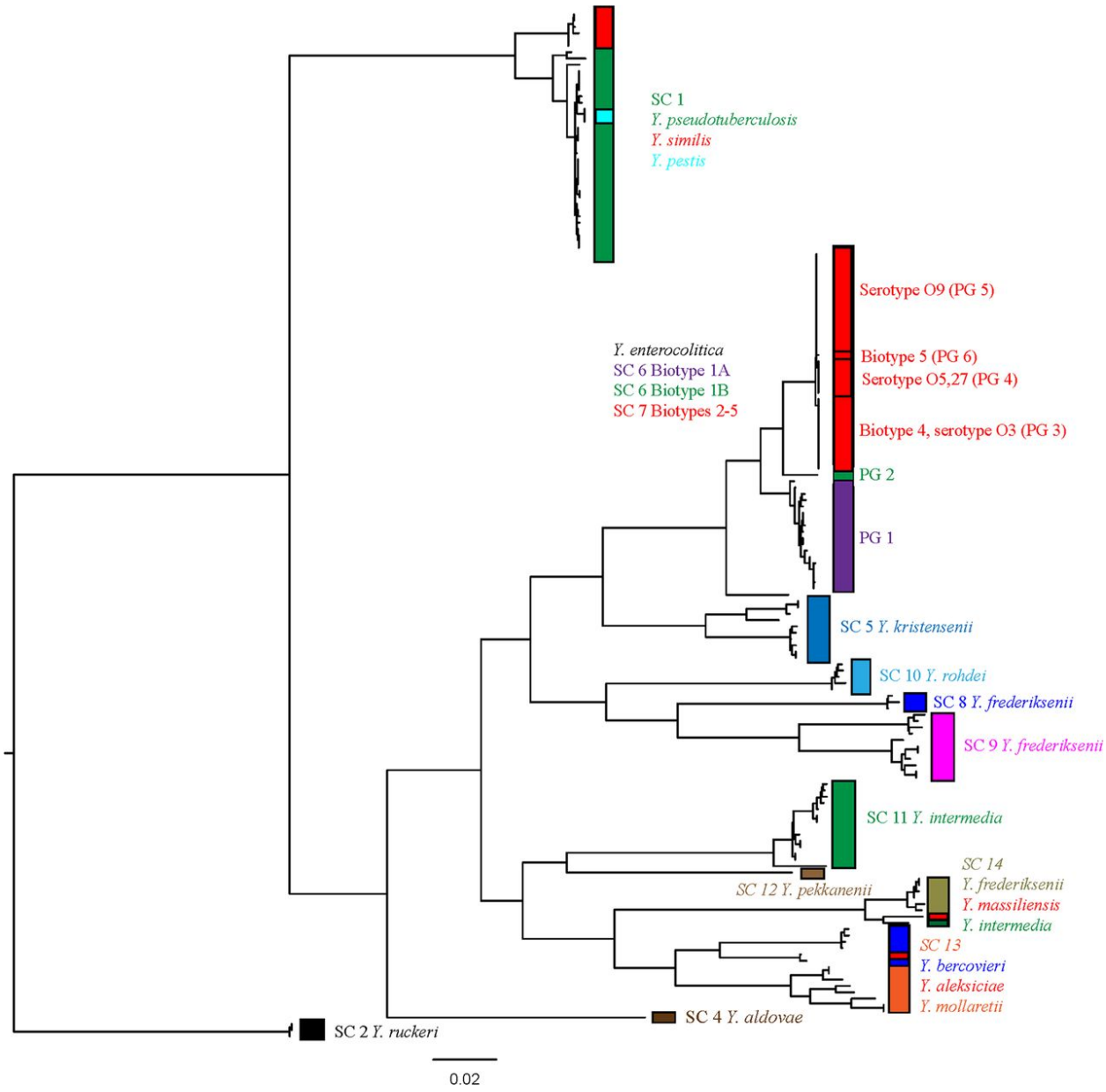

Figure 7. Maximum-likelihood phylogenetic tree of the genus *Yersinia* based on the concatenated sequences of 7 housekeeping genes (*aarF, dfp, galR, glnS, hemA, spA, rfaE*). SC= species cluster, PG=phylogroup. (From Hall et al. 2015). Published with a license from Copyright Clearance Center.

Also the studies of Reuter et al. (2012), Rakin et al. (2012), and Howard et al. (2006), support our finding that biotype 1A strains form a separate genomic group. Based on these results, biotype 1A strains considered non-pathogenic should not be included in subspecies *palearctica* containing low-pathogenic strains of biotypes 2–5.

5.4 Study IV: Identification of *Yersinia enterocolitica* 4/O:3 in cold-stored modified atmosphere-packed pig cheek meat

In total, 170 oxidase-negative isolates from violet red bile agar with glucose (VRBG) were studied by HindIII ribotyping. Of these, 105 were identified as *Y. enterocolitica* subsp. *palearctica*, and all originated from cheek meat after 12 days of storage at 6°C (Table 5). In the numerical analysis of the HindIII ribopatterns, the 105 isolates shared a similar pattern with *Yersinia enterocolita* subsp. *palearctica* type strain DSMZ 13030^{T}, and formed a unique cluster among the genus (Figure 1 publication IV). The bioserotype was confirmed as 4/O:3 by bio- and serotyping at least one isolate per sample. All isolates tested were *virF* and *ail* positive.

Table 5. Number of isolates identified as *Yersinia enterocolitica* subsp. *palearctica* 4/O:3 by 16S and 23S rRNA gene RFLP

Sample	Day	*Enterobacteriaceae*		*Y. enterocolitica* subsp. *palearctica*	
		No. of pos. samples	No. of isolates	No. of pos. samples	No. of isolates
Cheek	1	8	9	0	0
	13	20	109	20	105
Leg	1	0	0	0	0
	13	7	52	0	0
All samples		35	170	20	105

Table 6. shows the mean number of *Enterobacteriaceae* (purple colonies, oxidase negative on VRBG) and *Y. enterocolitica* bioserotype 4/O:3 in the beginning and the end of the 12 day storage of both studied types of pork meat strips. In both cheek and leg meat, the initial contamination of *Enterobacteriaceae* and *Y. enterocolitica* 4/O:3 was low. After the storage, *Y. enterocolitica* 4/O:3 was isolated from all cheek meat samples with the mean number 4.1 log cfu/g. The pH of the cheek meat was initially higher than that of hind leg meat and remained higher.

Table 6. Mean number of bacteria log cfu/g on pork strips packaged under modified atmosphere (30% O_2/70% CO_2) at 6°C

Sample	Cheek		Leg	
Day	1	13	1	13
No. of samples	20	20	20	20
pH (mean)	6.3	6.1	5.7	5.7
Mean number of bacteria on VRBG	1.6	4.5	1.7	1.3
Mean number of oxidase negative bacteria on VRGB	0.9	4.1	nd	1.3
Mean number of *Y. enterocolitica* 4/O:3 on VRBG	nd	4.1	nd	nd

nd=non detectable

Y. enterocolitica is psychrotrophic and it can grow at temperatures approaching 0°C (-2-45°C). The bacterium tolerates freezing. (Kapperud 1991, de Fernando et al. 1995). *Y. enterocolitica* has been shown to multiply in vacuum-packaged and modified-atmosphere-packaged (MAP) food (Kapperud 1991, Doherty et al. 1995). MAP and vacuum packaging are widely used in the meat industry to extend the shelf life of raw meat (Zhou et al. 2010). Most commonly, raw red meat (pork, beef) is packaged with a modified atmosphere (MA) of 70-80% O_2 and 20-30% CO_2 (McMillin 2008). CO_2 inhibits the growth of aerobic bacteria causing meat spoilage. The influence of carbon dioxide is increased at low temperatures, as the solubility of CO_2 to the product increases. (Gill & Tan 1980). Study IV shows that *Y. enterocolitica* may cause a risk in meat stored in MA (30% CO_2, 70% O_2). The initial number of *Y. enterocolitica* bioserotype 4/O:3 in pig cheek meat is affected by the slaughtering procedure, which frequently causes contamination of *Y. enterocolitica* from pigs tonsils (Fredriksson-Ahomaa et al. 2007, Bonardi et al. 2014). Pig cheek meat is considered a gourmet food. The finding that *Y. enterocolitica* bioserotype 4/O:3 can increase in MAP cheek meat from a non-detectable level to a level above 10^4 cfu/g is important in terms of preventing this pathogenic *Y. enterocolitica* bioserotype to cause food poisoning. The growth of *Y. enterocolitica* bioserotype 4/O:3 in refrigerated MAP cheek meat causes this food product to be high risk. Thus, pig head meat should not be packaged under MA.

5.5 Identification methods used

5.5.1 Phenotyping

Enterobacteriaceae are commonly identified by biochemical reactions using commercial tests such as the API 20 E strip tests (bioMérieux) and the Enterotube test (BBL DL Diagnostics) (Smith et al. 1972). These tests can cause false results when identifying *Yersinia* species. Many species show identical biochemical profiles. The culturing conditions and the instability of biochemical reactions may also affect the results. For example, *Y. frederiksenii* and *Y. intermedia* sometimes present an identical biochemical profile when using these tests. (Hao et al 2016). *Y. aldovae, Y. aleksiciae*, *Y. mollaretii*, *Y. bercovieri*, *Y. rohdei* and *Y. massiliensis* are often incorrectly identified as *Y. enterocolitica* (Sihvonen et al. 2009, Stephan et al. 2014). Especially, *Y. mollaretii, Y. bercovieri* and *Y. massieliensis* are incorrectly identified because they have identical API20E profiles with *Y. enterocolitica* (Merhej et al. 2008). It is also extremely difficult to distinguish *Y. similis, Y. wautersii* and *Y. pekkanenii* from *Y. pseudotuberculosis.* (Sprague et al. 2008, Murros-Kontiainen et al. 2011b, Savin et al. 2014). In the study of *Y. pekkanenii,* the strains Å125KOH2, ÅYV25K1 and ÅYV7.KOH2^{T} had been incorrectly identified as *Y. pseudotuberculosis* by phenotypic tests in a previous study by Niskanen et al. (2009). Their API 20E identification was 98.4% *Y. pseudotuberculosis*. *Y. nurmii* strains APN5b-c, ABF6a-c and APN3a-c^{T} were identified as 99.7% *Cedecea devisae*.

5.5.2 Multilocus sequence analysis

The MLSA method described by Kotetishvili et al. in 2005 was employed in this thesis to study phylogenetic relationships between *Yersinia* species. Figure 8 is a combination of data from publications I, II and III, and it shows a neighbor-joining tree based on the concatenated *glnA*, *gyrB*, *recA* and *HSP60* gene sequences of *Y. nurmii, Y. pekkanenii,* one European *Y. enterocolitica* 1A strain HYE1980 and *Yersinia* type/reference strains (Table 7). Gene sequences of *Y. entomohpaga*, *Y. massiliensis*, *Y. pestis* Antiqua and *Y. wautersii* were unavailable during the publication of *Y. nurmii* and *Y. pekkanenii,* but were obtained from whole genome sequences and also included in this figure. In the phylogenetic analysis of individual or concatenated housekeeping genes, performed by both the neighbor-joining, or maximum-parsimony methods, the strains of *Y. nurmii* and *Y. pekkanenii* formed monophyletic groups that were separate from other *Yersinia*. This MLSA method worked very well for the identification of *Yersinia* species, and also for differentiating non-pathogenic and pathogenic biotypes of *Y. enterocolitica*.

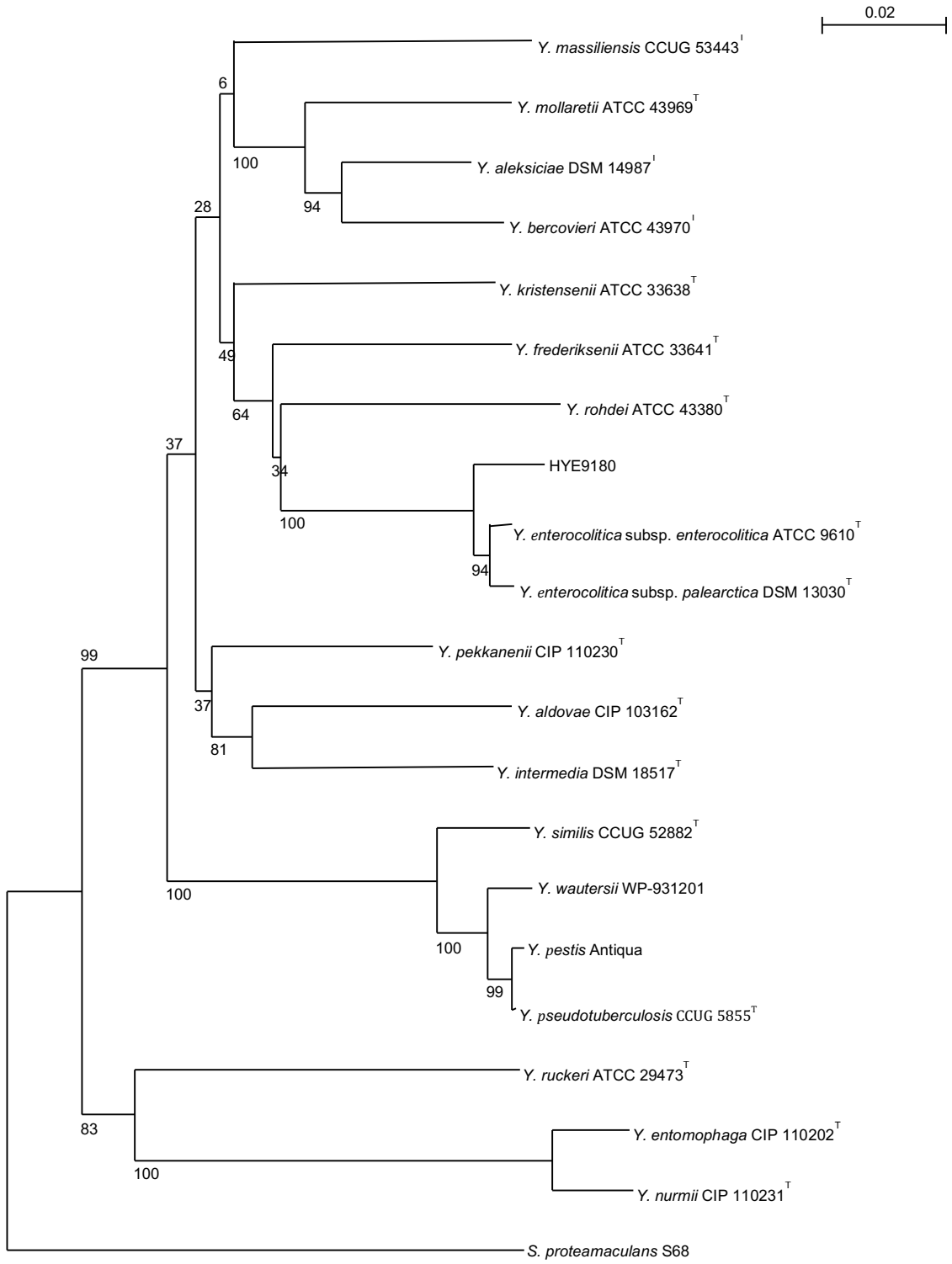

Figure 8. Neighbor-joining tree based on concatenated *glnA, gyrB, HSP60* and *recA* gene sequences of *Yersinia nurmii*, *Y. pekkanenii*, a European *Y. enterocolitica* biotype 1A strain (HYE9180), and type and reference strains of the genus *Yersinia*. Bootstrap values (%) for 1000 replicates are given at branch points. The bar represents 2% difference in nucleotide sequence. *S. proteamaculans* was used as outgroup. Gene bank accession numbers are presented in Table 7.

5.5.3 16S rRNA gene analysis

In the phylogenetic analysis of the 16S rRNA gene performed by both the neighbor-joining, or maximum-parsimony methods, the strains of *Y. nurmii* and *Y. pekkanenii* formed their own groups. Figure 9 shows a phylogenetic tree based on the 16S rRNA gene sequences (Table 7) of different *Yersinia* species calculated using the neighbor-joining method. *Y. pekkanenii* was most closely related to *Y. aldovae* and *Y. mollaretii,* and *Y. nurmii* to *Y. entomophaga*. In the study of the taxonomy of the European *Y. enterocolitica* 1A strains, the 16S rRNA analysis demonstrated insufficient discriminatory power to study the phylogenetic relationships between non-pathogenic biotype 1A strains and pathogenic biotypes 2 to 5 in the subsp. *palearctica*. A non-pathogenic European *Y. enterocolitica* 1A strain HYE9180 is included in Figure 9.

The 16S rRNA gene sequences of *Yersinia* species may show high similarity. Identical sequences can be found between strains of different species. Commonly the similarity values are as high as 96.9-99.8% between *Yersinia*. Therefore, the resolution power of the 16S rRNA gene has limitations for species delineation of *Yersinia.* (Hao et al. 2016).

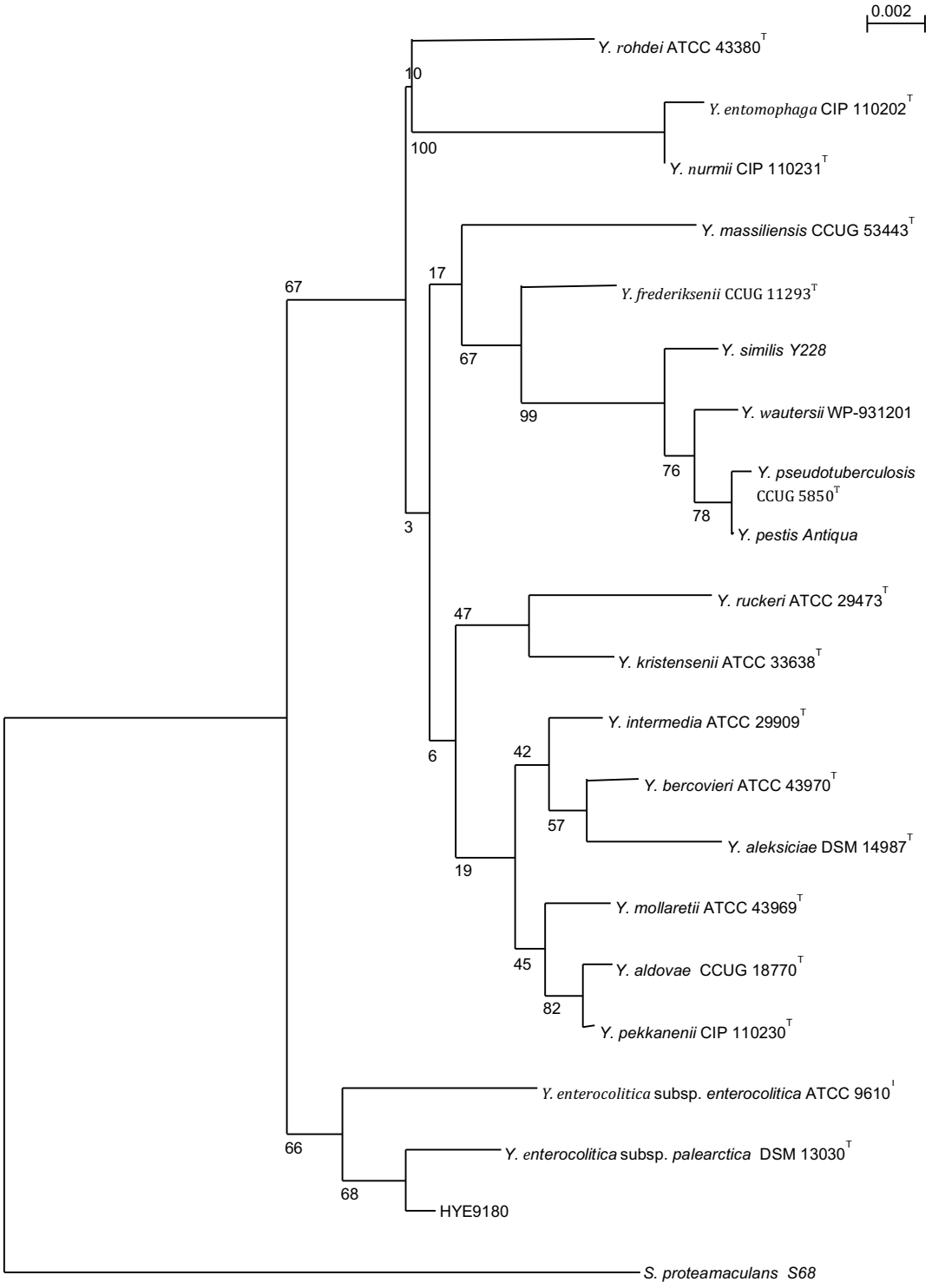

Figure 9. Neighbor-joining tree based on 16S rRNA gene sequences of *Yersinia nurmii*, *Y. pekkanenii*, a European *Y. enterocolitica* biotype 1A strain (HYE9180), and type and reference strains of the genus *Yersinia*. Bootstrap values (%) for 1000 replicates are given at branch points. The bar represents 2% difference in nucleotide sequence. *S. proteamaculans* was used as outgroup. Gene bank accession numbers are presented in Table 7.

Table 7. Strains used for the concatenated *glnA*, *gyrB*, *HSP60,* and *recA* gene (Concatenate strain) and 16S rRNA analysis (16S rRNA strain) in Figures 8. and 9. Gen Bank accession numbers presented

Species	Concatenate strain	*glnA* accession	*gyrB* accession	*HSP60* accession	*recA* accession	16Sr RNA strain	16S rRNA accession
Y. aldovae	CIP 103162 ^{T}	FJ717351	FJ717364	FJ717368	FJ717387	CCUG 18770 ^{T}	EF179125
Y. aleksiciae	DSM 14987 ^{T}	FJ717355	FJ717360	FJ717369	FJ717391	DSM 14987 ^{T}	FJ717341
Y. bercovieri	ATCC 43970 ^{T}	AY333017	AY332909	AY332858	AY332961	ATCC 43970 ^{T}	AF366377
Y. enterocolitica subsp. *enterocolitica*	ATCC 9610 ^{T}	FJ717353	FJ717366	FJ717378	FJ717388	ATCC 9610 ^{T}	FJ717343
Y. enterocolitica subsp. *palearctica*	DSM 13030 ^{T}	FJ717354	FJ717367	FJ717373	FJ717389	DSM 13030 ^{T}	FJ717344
Y. enterocolitica	HYE9180	KM888032	KM888024	KM888027	KM888033	HYE9180	KM888038
Y. entomophaga	CIP 110202 ^{T}	ERX139804	ERX139804	ERX139804	ERX139804	CIP 110202 ^{T}	ERX139804
Y. frederiksenii	ATCC 33641 ^{T}	AY333029	AY332923	EF173174	AY332975	CCUG 11293 ^{T}	EF179122
Y. intermedia	DSM 18517 ^{T}	FJ717352	FJ717363	EF173168	FJ717386	ATCC 29909 ^{T}	AF366380
Y. kristensenii	ATCC 33638 ^{T}	AY333044	AY332937	AY332883	AY332989	ATCC 33638 ^{T}	AF366381
Y. massiliensis	CCUG 53443 ^{T}	FJ717356	EF175588	EF173172	FJ717390	CCUG 53443 ^{T}	EF179119
Y. mollaretii	ATCC 43969 ^{T}	AY333050	AY332943	AY332889	AY332995	ATCC 43969 ^{T}	AF366382
Y. nurmii	CIP 110231 ^{T}	FJ717347	FJ717358	FJ717377	FJ717381	CIP 110231 ^{T}	FJ717338
Y. pekkanenii	CIP 110230 ^{T}	GQ451993	GQ451996	GQ451999	GQ452002	CIP 110230 ^{T}	GQ451990
Y. pestis Antiqua	Antiqua	CP000308	CP000308	CP000308	CP000308	Antiqua	CP000308
Y. pseudotuberculosis	CCUG 5855 ^{T}	FJ717349	FJ717361	FJ717370	FJ717384	CCUG 5855 ^{T}	FJ717342
Y. rohdei	ATCC 43380 ^{T}	AY333058	AY332948	AY332895	FJ717383	ATCC 43380 ^{T}	AF366384
Y. ruckeri	ATCC 29473 ^{T}	FJ717348	FJ717365	FJ717374	FJ717382	ATCC 29473 ^{T}	AF366385
Y. similis	CCUG 52882 ^{T}	FJ717350	FJ717362	FJ717371	FJ717385	Y228 ^{T}	AM182404
Y. wautersii	WP-931201	CVMG01000018	CVMG01000018	CVMG01000018	CVMG01000018	WP-931201	CVMG01000018
S. proteamaculans	568	CP000826	CP000826	CP000826	CP000826	568	CP000826

5.5.4 The 16S and 23S gene RFLP analysis

Ribopattern analysis clearly distinguished *Yersinia* species from each other. Figure 10 shows the HindIII ribopatterns of *Yersinia* type strains excluding *Y. pestis*. In the study of European *Y. enterocolitica* 1A strains, the biotype 1A strains formed a separate cluster from pathogenic biotypes. The results of the 16S and 23S gene RFLP analysis were congruent with the data obtained by the MLSA method used. In the study of the identification of *Yersinia enterocolitica* 4/O:3 in cold-stored modified atmosphere-packed pig cheek meat, *Y. enterocolitica* strains of bioserotype 4/O:3 could be identified by their unique ribopattern.

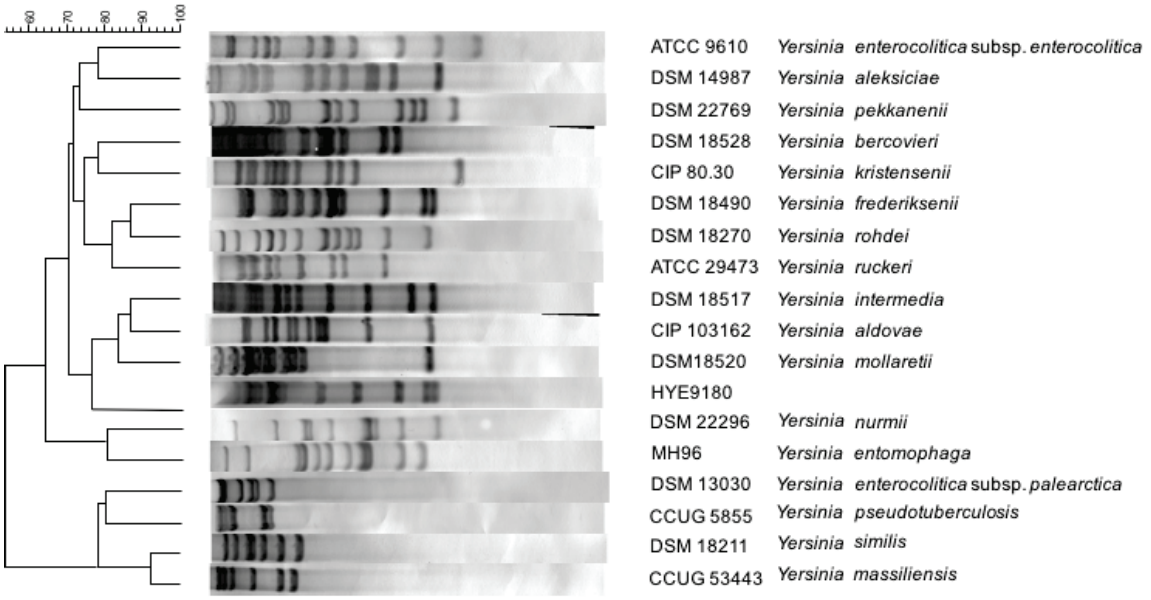

Figure 10. HindIII ribopatterns of *Yersinia* type strains and a European *Y. enterocolitica* 1A strain HYE9180. The numerical analyses of the patterns are presented as dendrograms. The molecular masses of the bands range from 1kb (right side) to 23 kb (left side). Dendrograms were calculated with the Dice coefficient using the UPGMA clustering method.

6 CONCLUSIONS

A number of key findings have derived from this investigation

1. Strains APN5b-c, ABF6a-c and APN3a-c^T isolated from three packages of broiler meat cuts, packaged under modified atmosphere represent a novel species *Y. nurmii* sp. nov.

2. Strains Å125KOH2, ÅYV25K1 and ÅYV7.KOH2^T, originating from water, soil and lettuce samples, represent a novel species *Y. pekkanenii* sp. nov.

3. European non-pathogenic *Y. enterocolitica* 1A strains form their own genomic group with indication of the existence of a subspecies formed by *Y. enterocolitica* biotype 1A strains.

4. *Y. enterocolitica* subsp. *palearctica* bioserotype 4/O:3 was detected in high numbers in pig cheek meat packaged under modified atmosphere (30% CO_2 /70% O_2) at 6°C.

5. The 16S and 23S rRNA gene RFLP using the HindIII enzyme identified *Yersinia* at the species level and also separated non-pathogenic European *Y. enterocolitica* strains into an own cluster. The results were in congruence with the results of the MLSA analysis using housekeeping genes *glnA*, *gyrB*, *recA* and *HSP60*. Phenotypic methods and 16S rRNA gene analysis cannot alone be used in the identification of *Yersinia* species.

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