OSAMA SAMMRA

Phenotypic and genotypic characteristics of bacteria of genus *Arcanobacterium* with the emphasis on the characterization of four newly described *Arcanobacterium* species

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eingereicht von

Osama Sammra
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Dekan: Prof. Dr. Dr. h.c. Martin Kramer

Erster Gutachter: Prof. Dr. Christoph Lämmler

Zweiter Gutachter: Prof. Dr. Michael Bülte

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Keep your dreams alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination, and dedication. Remember all things are possible for those who believe….. Gail Devers

This work is dedicated

to my

beloved parents and family

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Osama Sammra

Abbreviations

A.	*Arcanobacterium*
A.	*Actinomyces*
AFLP	Amplified fragment length polymorphism
ANI	average nucleotide identity
Bp	base pair
C	cytosine
CAMP-Test	Christie-Atkins-Munch-Peterson-Test
CDC	cholesterol-dependant cytolysin
CFA	cellular fatty acid
cpn60	heat shock protein (chaperonin) encoding gene
CO_2^-	carbon dioxide
Da	Dalton, unit of molecular mass
DDH	DNA-DNA hybridization
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Microorganismen und Zellkulturen
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
et al.	et alii (and others)
fig.	figure
FT-IR spectroscopy	Fourier Transform Infrared Spectroscopy
g	gram
G	Guanine
gap	glyceraldehyde 3-phosphate dehydrogenase encoding gene
GC	gas chromatography
Gen. n.	genus novus or new genus
h	hour
ILY	Intermedilysin from *Streptococcus intermedius*
ily	*Streptococcus intermedius intermedilysin* encoding gene
ISR	16S-23S rDNA intergenic spacer region
kb	kilobase
kDa	kilodalton
kg	kilogram
L	liter
LLO	Listeriolysin O from *Listeria monocytogenes*
llo	*Listeria monocytogenes* listeriolysin encoding gene
LPS	lipopolysaccharides
mA	milliampere
MALDI-TOF MS	matrix-assisted laser desorption time of flight mass spectrometry

MK	menaquinones
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
N	Normality
NCBI	National Center for Biotechnology Information
mg	milligram
min	minute
mm	millimeter
mmol	millimole
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
mol	amounts of a chemical substance
MS	mass spectrometry
MSP	main spectrum (reference spectrum of the peptidic raw spectra)
nt	nucleotide
N.L.	new latin, i.e., latinate phrases coined for scientific terms
P	pico (10^{-12})
PCR	polymerase chain reaction
PFO	perfringolysin O from *Clostridium perfringens*
pfo	*Clostridium perfringens* perfringolysin encoding gene
phl	*Arcanobacterium phocae* phocaelysin encoding gene
pld	*Arcanobacterium haemolyticum* phospholipase D encoding gene
PLO	Pyolysin from *Arcanobacterium* (*Trueperella*) *pyogenes*
plo	*A* (*T.*) *pyogenes* pyolysin encoding gene
PLY	Pneumolysin from *Streptococcus pneumoniae*
ply	*Streptococcus pneumoniae* pneumolysin encoding gene
pmol	picomole
RAPD	Random amplified polymorphism DNA
RBR	relative binding ration
Rep-PCR	Repetitive sequence-based PCR
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpoB	beta subunit of the RNA polymerase encoding gene
s	second
SLO	Streptolysin O from *Streptococcus pyogenes*
slo	*Streptococcus pyogenes* streptolysin encoding gene
sodA	superoxidase dismutase A encoding gene
T	Thymin
T.	*Trueperella*
Tab.	Table
tuf	elongation factor tu encoding gene
U	unit (international unit)
UV	ultraviolet
V	volt
Vol.	volume
ºC	degree Celsius

µg	microgram
µl	microliter
µm	micrometer

Table of Content

Chapter 1 - Introduction

Genus *Arcanobacterium (A),* belonging to the family *Actinomycetaceae*, was first described by Collins et al. (1982b) for the gram-positive, facultative anaerobic, asporogenic bacterium *A. haemolyticum,* previously known as *Corynebacterium haemolyticum* that was originally isolated from infected wounds of American soldiers (MacLean, 1946). Few years later, *Arcanobacterium pyogenes* and *Arcanobacterium bernardiae* replaced *Actinomyces pyogenes* and *Actinomyces bernardiae*, respectively (Ramos et al., 1997). Furthermore, six new species namely *Arcanobacterium phocae, Arcanobacterium pluranimalium, Arcanobacterium hippocoleae, Arcanobacterium bialowiezense, Arcanobacterium bonasi* and *Arcanobacterium abortisuis* were described by Ramos et al. (1997), Lawson et al. (2001), Hoyles et al. (2002), Lehnen et al. (2006) and Azuma et al. (2009), respectively. In the following years, Yassin et al. (2011) proposed, after comparative chemotaxonomic and phylogenetic studies, that genus *Arcanobacterium* should be restricted to *A. haemolyticum, A. phocae, A. pluranimalium* and *A. hippocoleae* and that *A. pyogenes*, *A. bernardiae, A. bonasi* and *A. bialowiezense* should be reclassified into the newly described genus *Trueperella* (*T*). Bacteria of genera *Arcanobacterium* and *Trueperella* are well known to cause infections in both animals and humans.

The aim of the present study was to identify and further characterize bacteria of genus *Arcanobacterium,* namely *A. haemolyticum* of various origins, *A. pluranimalium* of ovine and bovine origin and from a juvenile giraffe, the hitherto not described novel species *A. canis* and *A. phocisimile*, *A. pinnipediorum* and *A. wilhelmae* isolated from a dog, harbor seals, and a rhinoceros, respectively, using traditional and molecular methods. The bacteria, also including reference strains of genera *Arcanobacterium* and *Trueperella,* were investigated phenotypically, by MALDI-TOF MS and genotypically by investigating various species-specific targets and by studying potential virulence factor encoding genes.

Chapter 2 - Review of literature

2.1 - Polyphasic taxonomy

2.1.1 - Phenotypic characterization

2.1.1.1 - Colony characterization, biochemical and physiological analysis

The first successful approach for growing and sub-culturing of bacteria on a solid media was accomplished and disclosed by Robert Koch during the international medical congress held in London in 1881 (Koch, 1881). In the following year, this nutrient media underwent a critical change by replacing gelatin with the more heat-stable 'agar'. Later, Friedrich Loeffler improved the formula by introducing the amino-nitrogen rich peptone and high osmolarity salt (Loeffler, 1885). However, the plate was remodeled in 1887 by Julius Richard Petri, to follow his name 'Petri dish' and acquire the shape and design known today (Petri, 1887).

Characterization of bacterial colony morphology is a fundamental classification technique that relies on the investigation of bacterial traits, growth requirements as well as biochemical and physiological analysis by embracing macro- and microscope-dependent methods (Jackman, 2012; Li et al., 2016). Macroscopic or gross morphological investigation includes exploration of colony shape, size, margins, pigmentation (chromogenesis), opacity, elevation, surface and consistency.

Shape: bacterial colonies are observed as rod-shaped (bacillus), spherical (coccus), spiral or elongated cocci (coccobacilli), long threads (filamentous), slightly curved rods (vibrios), tapered ends (fusiform) and pleomorphic (e.g. *Mycoplasma pneumoniae*).

Types of cocci: coccal bacteria exist either in pairs (diplococci) such as *Streptococcus pneumoniae*, in chains as *Streptococcus agalactiae*, in four-cell packets (tetrads) like *Tetragenococcus* spp., cuboidal form (e.g *Sarciniae*) or in grape-like clusters (staphylococci) such as *Staphylococcus aureus*.

Types of bacilli: single bacilli (e.g. *Bacillus cereus*), diplo-bacilli (e.g. *Klebsiella pneumoniae*), streptobacilli (e.g. *Streptobacillus moniliformis*), cocco-bacilli (e.g. *Haemophilus influenzae*) and palisades (e.g. *Corynebacterium tuberculostearicum*).

Types of spiral forms of bacteria: vibrio (e.g. *Vibrio cholera*), spirilla (e.g. *Campylobacter coli*) and spirochetes (e.g. *Leptospira canicola*).

Elevation: are detected through a cross-sectional view of the bacterial colonies. Bacteria are viewed as raised, convex, flat, umbonate or cateriform.

Margins: focusing on the shape of the edges of the colony and appear as entire, undulate, filiform, curled and lobate.

Surface: refers to smooth, rough, glistening, dull, and wrinkled.

Opacity: transparent (clear), opaque, translucent (semi-clear with distorted vision), iridescent etc.

Pigmentation: white, buff, purple, red.

Spherial bacteria measure between 0.5-2.0 μm, whereas, the size of rod-shaped and filamentous bacteria range between 1.0-10.0 μm and 0.25-1.0 μm, respectively.

Microbial cells are investigated microscopically by the detection of Gram reaction, shape, conformation (single, chain), existence and characteristics of endospores (central, terminal), type of flagella (polar, peritrichous) (Brenner et al., 2005).

Gram stains (Gram, 1884) are made up of a solvent and a benzene derivative colored structure known as the chromogen. The charging element of the chromogen is the autochrome, which allows staining of the cells by forming an ionic or covalent bond. The autochrome becomes positively charged by donating a hydroxide ion (OH^-) or receiving a hydrogen ion (H^+) and is therefore, appealed to the negative charge on most bacterial cells leading to their staining. The most commonly used basic (alkaline) stains are: methylene blue, crystal violet and carbol fuchsin or safranin.

Gram staining is widely used to discriminate bacteria into Gram-positive and Gram-negative, thus endowing a better specification of their cell morphology, size, and provision. Both Gram-positive and Gram-negative bacteria are primarily stained with crystal violet followed by applying iodine as a 'mordant' which catalyzes staining by creating a crystal-violet-iodine complex. A de-coloration step is followed, by applying an alcohol or acetone reagent. Gram-negative bacteria have a lipid-rich cell wall and will be degraded by the decolorizing reagent, increasing porosity and thereby losing the crystal violet-iodine complex. Contrariwise, the thick peptidoglycan cell wall of Gram-positive bacteria containing a strong cross-linking network interconnected by teichoic acid resists discoloration and retain the color of the crystal violet-iodine complex. Gram-negative bacteria are counter-stained by safranin giving it a pink-red color while Gram-positive bacteria appear violet or blue (Beveridge, 2001; Leboffe and Pierce, 2011).

Numerous biochemical and physiological analysis techniques are being applied for the identification of bacteria. These methods can be classified into:

Rapid biochemical reactions: relies on investigating the reaction of particular bacterial enzyme(s) and could be immediate or might require incubation for few hours. This category includes catalase, oxidase, amylase, glucosidase, urease, gelatinase and others.
Fermentation of carbohydrates: investigates the ability of bacteria to utilize carbohydrates as a sole carbon source, such as glucose, lactose, maltose, mannitol and others.
Other physiological properties affecting growth conditions such as temperature, pH, salt concentration and antibiotic resistance (Yousef, 2008).
Species of genus *Arcanobacterium* are described as Gram-positive, facultative anaerobic, non-sporulating bacteria measuring 0.3-0.8 x 1.0-5.0 µm. According to various authors, the young bacterial colonies appear as slender irregular bacillary to V-shaped that tend to be irregular coccoid when mature. For optimal growth, bacteria of genus *Arcanobacterium* are incubated on 5 % sheep blood agar plates for 48 h at 37 ºC in a CO_2^- enhanced atmosphere and display a complete zone of hemolysis (Collins et al., 1982b; Schaal et al., 2006; Yassin et al., 2011). The bacteria become inactive when exposed to a temperature of 60 ºC for 15 min (Collins et al., 1982b).

2.1.1.2. - Numerical taxonomy

Numerical methods for taxonomic classification of gram positive-bacteria were first developed by Sokal and Sneath in 1963. This technique analyses a large-scale of phenotypic and genotypic data based on the similarity between selected microorganisms using a specially designed computer software. The procedure acts by appointing a numeral code to a set of phenotypic and genotypic characters of a certain microorganism scaled in a recognizable format. The correlation between these characters is laid out by various algorithms yielding a similarity matrix that is used to build up a taxonomic structure (e.g. by cluster analysis), thus forming phylogenic tree-like diagrams (dendrograms). These trees display the complete pattern of similarities and discrepancies of the target microorganism (Sneath, 1995; Sarethy et al., 2014).

2.1.1.3 - Chemotaxonomic analysis

2.1.1.3.1 - Peptidoglycan analysis

The bacterial cell is enclosed by a cell envelope consisting of a cell membrane and a cell wall. The cell wall is a stiff structure encompassing the bacterial cell giving it rigidity and providing it with protection against any physical thrust. It almost exists in all bacterial genera with some

exceptions such as in *Mycoplasma* (Ryan and Ray, 2004). The cell wall of Gram-negative bacteria consists of a thin peptidoglycan layer enclosed by an outer membrane comprehending lipopolysaccharide. Gram-positive bacteria contain no outer membrane but are embodied by a thick layer of peptidoglycan (murein) forming a mesh-like layer interlaced with outstretched teichoic acid polymers (Silhavy et al., 2010).

Gram-negative bacteria bear a symmetric peptidoglycan structure (Schleifer and Kandler, 1972) in contrast to a heterogenic structure in Gram-positive bacteria making it a significant taxonomic tool for the differentiation of the latter. The peptidoglycan constitutes around 30-70 % of the Gram-positive bacterial cell wall, such as in staphylococci and streptococci, in addition to polysaccharides and teichoic acids and less than 10 % of Gram-negative cell walls as in *E. coli*, which consists additionally of lipopolysaccharides and lipoproteins (Schumann, 2011).

In 1972, a review on the relevance of peptidoglycan diversity for bacterial taxonomy was established by Schleifer and Kandler. Furthermore, the analysis of the peptidoglycan structure became a mandatory criterion demanded for describing all members of Gram-positive new taxa (Tindal et al., 2010). A detailed understanding of the analytical approaches of peptidoglycan structures including information from whole-cell hyrdrolysates and preparation and analyses of peptidoglycan was described by Schumann (2011). Since its first description, peptidoglycan has been intensively investigated and considerably used as a critical taxonomic marker in the study of genus *Actinomyces* (Zavadova et al., 1973), for the differentiation of various species of family *Bifidobacteriaceae* and discriminating between different subspecies of genus *Bifidobacterium* (Mattorelli and Sgorbati, 2017). More recently, based on the variation in the peptidoglycan cell wall components of species of family *Opitutaceae*, three novel species of genus *Lacunisphaera* could be successfully identified by Rast et al. (2017).

2.1.1.3.2 - Fatty acid analysis

Cellular fatty acid (CFA) analysis is one of the most precise method used in chemotaxonomy (Tornabene, 1985). Initial CFA analysis attempts for the identification of bacteria were brought about in the 1950s (James and Martin, 1952). However, the first successful bacterial identification using CFA analysis was carried out by Abel et al. (1963). A diversity of lipids are available in the bacterial cells. The main components of the lipid bilayer of the bacterial cell membranes are the polar lipids which have been investigated for classification and characterization by Jones and Krieg (1984). The fatty acid cell layer consists mainly of

lipopolysaccharides (LPS) in gram-negative bacteria and of lipoteichoic acid in gram-positive bacteria. One of the most conserved CFA in prokaroytes is the 16-carbon saturated CFA hexadeconic acid, nonetheless, more than 300 CFAs and relevant compounds have already been determined and used for quantitative and qualitative differentiation (Sasser, 1990; Welch, 1991; Suzuki et al., 1993). The variation in fatty acid composition based on the length of chain, position of the double bond and other substituent groups was of great advantage in bacterial taxonomy (Suzuki et al., 1993). Fatty acids and glycerol-linked esters are relatively common components of all bacteria and could be used as a constant parameter, however, the bacteria need to be cultured under standard conditions. Despite acquiring an incomplete database library, the Sherlock MIS System (MIDI Inc.) remains a broadly used, accurate and low cost system for the identification of microorganisms by analyzing extracted fatty acid methyl esters (FAMEs) using a fully automated gas chromatographic (GC) system (Vandamme et al., 1996; Tindal et al., 2010; Kunitsky et al., 2006).

2.1.1.3.3 - Isoprenoid quinones

Isoprenoid quinones, known also as prenylquinones (Lichtenthaler et al., 1977; Goodwin et al., 1977) are imperial cytoplasmic membrane-attached compounds that exist in most prokaryotes with an exception of some compulsory fermentative bacteria and Methanosarcinales (Collins et al., 1981; Beifuss et al., 2005). They are involved in vital cellular activities such as transport of electrons, oxidative phosphorylation, in active transport and conjointly associating in the gene expression regulation and cell signaling (Vandamme et al., 1996; Kawamukai et al., 2002; Nowicka and Kruk, 2010). The two eminent groups of isoprenoid quinones are the naphthoquinones (divided into the sub-groups phylloquinone and menaquinone) and the benzoquinones which are classified based on their ring substitution form into ubiquinones, rhodoquinones and plastoquinones. (Nowicka and Kruk, 2010; Sarethy et al., 2014). The heterogeneity of its side chains, length and degree of saturation and moreover the existence of ancillary groups helped the isoprenoid quinones to become an important taxonomic tool for the characterization of bacteria (Nowicka and Kruk, 2010; Collins et al., 1981).

2.1.1.3.4 - Polyamines

Polyamines are a group of organic compounds that are present in most prokaryotes. The actual role polyamines play in bacterial cells is unclear, however, they appear to collaborate in the stability of the DNA framework and are involved in regulating osmotic pressure of the cell and

in expression of particular genes. Polyamines are useful bacterial chemotaxonomic biomarker prevailing in the cell envelope, cytoplasm and are believed to be connected to the DNA, RNA or ribosomal structures that are determined using gas chromatography or high-performance liquid chromatography (Busse and Auling, 1998; Busse, 2011; Sarethy et al., 2014). Polyamine arrays could be used for indicating relevance among prokaryotes up to the species level (Vandamme et al., 1996).

2.1.1.4 - Spectroscopy techniques

The use of proteomics in microbiology proved to be a fast, accurate and cost-effective tool for investigating and characterizing phenotypic properties of bacteria. The most prevailing methods used in this field include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Emerson et al., 2008) and Fourier-transform infrared spectroscopy (FT-IR) (Al-Qadiri et al., 2006; Emerson et al., 2008). Comparably, MALD-TOF MS analysis is capable of identifying bacteria to the species level by verifying bacterial protein molecular masses after a complete extraction of bacterial contents, whereas FT-IR spectroscopy relies on scrutinizing minor amounts of bacterial biomass and investigating diverse active groups including nucleic acids, polysaccharides, fatty acids and other biochemical molecules and structures without affecting the entire cellular component (Toyran et al., 2006; Garip et al., 2009; Lau et al., 2011).

2.1.1.4.1 - Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy (MALDI-TOF MS analysis)

Matrix-assisted Laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a simple, precise and cost-effective analytical technique used for the fast identification and characterization of microorganisms. Mass spectroscopy (MS) was first developed in the late 19th Century by J.J Thomson (1897) to calculate the ratio of mass/charge of electrons (Griffiths, 1997). However, the first use of MS technique for the ionization and analysis of large molecular masses ranging between 100 Da and 100 KDa was in 1988 (Karas and Hillenkamp). In the 1980's, the progression of soft ionization methods like electrospray ionization (ESI) and MALDI-TOF MS collaborated in the analysis of large protein biomolecules (Tanaka and Fenn, 2002). Meanwhile, the first description about the recovery of MALDI-TOF MS fingerprint spectra directly from entire bacterial cells was mentioned by Holland et al. (1996). In the year 2004 this technique was adopted as a regular criterion in

routine microbiology after an entire database for the identification of bacteria was completed by Keys and coauthors. Ahead of this date, a broad diversity of microorganisms were further characterized by the application of this technique such as, bacteria and Archaea (Seng et al., 2009; La Scola et al., 2010), viruses (Downard et al., 2009; Schwahn et al., 2009), molds (Alanio et al., 2010) and yeasts (Ferroni et al., 2010). Moreover, MALDI-TOF MS has become one of the predominantly used techniques for the analysis of bacteria due to its capability to instantly analyse the entire bacterial cell, as well as, its competence in producing well-monitored up-right spectra arrays, which comprise the essential data required for the identification and characterization of bacterial species. The analysis is attained by matching the spectra fingerprint outputs of anonymous species to values obtained from a built-in spectra library (Emerson et al., 2008). It was considerably found that the main protein mass spectra resulting from the analysis of the entire bacterial cells were ribosomal proteins (Suh et al., 2005; Maier and Kostrzewa, 2007).

The application of MALDI-TOF MS in classification of microorganisms and verification of virulence factors was further described by various authors (Bizzini and Greub, 2010; Murray, 2010).

The device consists of three basic units: i) the source of ions which is responsible for the ionization and transformation of the sample molecules (analyte) into the gaseous state, ii) the mass analyzer which separates the ions based on the ratio of their mass-to-charge (m/z) and iii) the detector which acts on scanning the separated ions.

The microbial sample (analyte) is mixed and embedded into a matrix which is low acidic in nature and acts on lysing the cellular envelope components. The form of matrix used depends on the microorganism as they vary in their cell envelope components; bacteria bears a peptidoglycan and glycerol-ester lipid structure compared to a pseudomureins and glycerol-ether lipids in Archaea (De Rosa et al., 1986; Koga and Morii, 2007). However, a frequently used matrix consists of α-cyano-4-hydroxycinnamic acid with 50% acetonitrile and 2.5 % trifluoroacetic acid (Dridi et al., 2011). The matrix is let to dry until forming crystals. It is then transferred into a mass spectrometer and is shot by a laser beam causing desorption and ionization of the biomolecules. The ions are sped up by an electrostatic field and forced out through a vacuum flight-tube reaching a detector at the tube terminal. The ions are separated based on their mass to charge ratio (*m/z*) where lighter ions reach faster to the detector (Fig. 1) forming a mass spectrum (Croxatto et al., 2012; Cobo, 2013).

$$tof = \alpha \sqrt{\frac{m}{z}}$$

Fig. 1: Time of flight (*tof*) formula where; (*α) stands for a constant factor, (m) for the mass and (z) for charge of molecules* (reformed in consonance with http://de.wikipedia.org/wiki/MALDI-TOF).

2.1.1.4.2 - Fourier Transform-Infrared Spectroscopy (FT-IR)

Infrared spectroscopy is a phenotypic cellular-based taxonomical technique that uses highly reproducible fingerprint-like patterns to differentiate between various bacteria up to the species level. Infrared spectra represent the unique cellular component for each bacterium and could be useful to identify different species by comparing generated patterns to a reference database spectra (Naumann, D, 1985; Naumann et al., 1988a, b; 1990; Helm et al., 1990). FT-IR spectroscopy has already been used for the identification of *Listeria* spp. (Holt et al., 1995), *Streptococcus* spp. (Goodacre et al., 1996), various species of genera *Bacillus* (Beattie et al., 1998, Lin et al., 1998), as well as for detection of bacteria causing urinary tract infections (Goodacre et al., 1998). More recently, this technique proved to be a useful tool for the identification of *T. pyogenes* strains isolated from bovine clinical mastitis (Nagib et al., 2014).

2.1.2 - Genotypic characterization

2.1.2.1 - Ribonucleic acid (RNA) as a molecular target

Ribosomes are actively pervasive units that have been early identified and analyzed by Carl Woese (Woese, 1987, 1990). The ribosomal RNA (rRNA) genes are found to be vital in all prokaryotic cells and contain highly conserved regions. Furthermore, the bacterial ribosome symbolized as '70S' (due to its sedimentation coefficient) is divided into two subunits; a large 50S subunit and a smaller 30S subunit (SSU). The former subunit comprises the 5S and 23S RNA whereas the latter contains the 16S RNA. In prokaryotes, these genes are systematically arranged together in the consequent order 16S-23S-5S to form an rRNA operon which acts as its basic transcription and functional unit (Gürtler and Stanisich, 1996; Pisabarro et al., 1998; Roth et al., 1998; Klappenbach et al., 2000). Moreover, it is assumed that these operons play a vital role in the bacterial growth rate (García-Martínez et al., 1999). The number of these

operons vary among different bacterial genomes and may range from a single copy such as in *Rickettsia prowazekii* (Anderson et al., 1995) and *Mycoplasma pneumoniae* (Bercovier et al., 1986) up to 15 as in *Clostridium paradoxum* (Rainey et al., 1996). Meanwhile, enteric bacteria *Escherichia (E.) coli* that has been used as a standard model for 16S rRNA studies in prokaryotes constitutes seven operons (Ellwood and Nomura, 1980).

2.1.2.1.1 - 16S rRNA gene

The development of polymerase chain reaction (PCR) by Kary Mullis in 1985, followed by the successful amplification and sequencing of the 16S rRNA gene, had underlined a dramatic transformation in the taxonomy of prokaryotes. Moreover, owing to its constitution of highly conserved and hyper variable regions, the 16S rRNA had emerged as a pivotal criterion and is currently reported as the 'golden standard' and the 'first-line tool' in taxonomic identification and classification of prokaryotes (Stackerbrandt et al., 2002; Rainey et al., 1996; Clarridge, 2004; Rosselló-Móra, 2005; Tringe and Hugenholtz, 2008; Sentausa and Fournier, 2013). The estimated size of the bacterial 16S rRNA is approximately 1550 bp (Wittmann, 1976). It incorporates nine hypervariable regions flanked by highly conserved regions which provide an optimal target for universal primers design (Baker et al., 2003; Rossi-Tamisier et al., 2015; Yang et al., 2016). Despite that fact that there is no specific common hypervariable region for all different bacteria (Chakravorty et al., 2007), it has been reported that the primal 500 bp stretch endures sufficient data for bacterial identification. Nevertheless, it has been found necessary to sequence the whole ~1500 bp length for specific bacterial strains as well as, for the characterization of novel species (Clarridge, 2004).

Furthermore, Stackebrandt and Goebel in 1994 had determined the cut-off values of 16S rRNA sequence similarity to be ≥ 97 % to define species and ≥ 95 % for genera. However, after further revision, the cut-off value at the species level was raised to ≥ 98.7 % (Stackebrandt and Ebers, 2006). It is noteworthy that these cut-off values do not conform to all genera, such as in genus *Bacillus* (99.5 % similarity between *B. globisporus* and *B. psychrophilus*) (Janda and Abott, 2007) and among the various species of genus *Rickettsia* with a sequence similarity of > 99 % (Fournier and Raoult, 2009; Rossi-Tasmisier et al., 2015). Hence, for an accurate consideration of these values, the sequence need to be of a high quality (<1 % unspecified bases) and with a nearly complete length coverage (a minimum of 1,000 bp) (Caumette et al., 2015). A list of widely used 16S rRNA primers showing their reciprocal positions on the *Escherichia coli* 16S

rRNA (based on the sequence obtained from GenBank; accession number [J01859]) are noted in **Table 1.**

There are three main curated databases to which the nucleotide sequences could be submitted; EMBL (Cochrane et al., 2008; http: //www.ebi.ac.uk), GenBank (Benson et al., 2011; http://www.ncbi.nml.nih.gov/genbank) and DDBJ (Kaminuma et al., 2011; http: //www.ddbj.nig.ac.jp). In addition, few other rRNA-specific databases exist such as the SILVA, RDP and Greegenes Projects (Ludwig et al., 2011).

The SILVA project (Pruesse et al., 2007; http://www.arb-silva.de) operates on the Eucarya, Archaea and Bacteria whereas the RDP (Cole et al., 2009; http://rdp.cme.msu.edu) and the Greengenes Projects (DeSantis et al., 2006; http://greengenes.lbl.gov) are mainly specific for rRNA sequences.

Table 1.

List of most commonly used 16S rRNA "universal" primers, described by various authors and their corresponding positions on the 16S rRNA gene of *Escherichia (E.) coli* (Brosius et al., 1981).

Primer	***Sequence***	***Position***	***Reference***
8F	5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3'	8-27	Turner et al. (1999)
27F	5'-AGA-GTT-TGA-TCM-TGG-CTC-AG-3	27-46	Lane et al. (1991)
357F	5'-CTC-CTA-CGG-GAG-GCA-GCA-G-3'	357-375	Turner et al. (1991)
533F	5'-GTG-CCA-GCA-GCC-GCG-GTA-A-3'	533-551	Weisburg et al. (1991)
519R	5'-GWA-TTA-CCG-CGG-CKG-CTG-3'	519-502	Turner et al. (1999)
907R	5'-CCG-TCA-ATT-CMT-TTR-AGT-TT-3'	907-888	Lane et al. (1991)
1391R	3'-GAC-GGG-CGG-TGT-GTR-CA-3'	1391-1375	Turner et al. (1999)
1410R	3'-GTG-TGA-CGG-GCG-GTG-TGT-AC-3	1410-1391	Kuhnert et al. (1996)
1492R	3'-GGT-TAC-CTT-GTT-ACG-ACT-T-3'	1492-1474	Turner et al. (1996)

2.1.2.1.2 - 16S-23S rDNA intergenic spacer regsion (ISR)

The 16S-23S rDNA intergenic spacer (ISR) region constitutes the DNA segment located between the 16S and 23S genes. It has also been termed 'internal transcribed spacer(s) (ITS) due to the transcription process occurring within this region in collaboration with the ribosomal genes (Osorio et al., 2005). It has been found that there is a substantial length variation of ISR among different species and equivalently between different operons of the same individual (Condon et al., 1995). This might be attributed to the existence of various functional units such as the tRNA genes (García-Martínez et al., 1999; Gürtler and Stanisich, 1996; Normand et al., 1996). By virtue of its position, lying between two highly conserved regions, which could be used as prominent primer-binding sites (Barry et al., 1991; Jensen et al., 1993; Kostman et al., 1995), ISR had become a remarkably useful tool for epidemiological and taxonomic studies of prokaryotes (Amann et al., 1995). Moreover, it has shown high efficiency in the characterization and differentiation of bacteria up to and below the species level (Jensen et al., 1993; Wang et al., 1997; Zhang et al., 1997; García-Martínez et al., 1999).

2.1.2.1.3 - 23S rRNA gene

The 23S rRNA is the larger component of the 50S rRNA subunit. It measures 2,904 bp in *E. coli* and is considered the largest functional unit in the rRNA operon. It resembles the 16S rRNA in having conserved and variable regions, yet with a comparably better phylogenetic resolution advancing from its greater length (Ludwig et al., 1994; Trebesius et al., 1994). Meanwhile, Hunt et al. (2006) had successfully developed a set of highly specific universal primers which could amplify a significant portion of the 23S sequence for different bacterial species. Formerly, there had been a bias in favor of 16S rRNA for taxonomic classification, however, a re-evaluation of the use of 23S rRNA following the development in whole-genome sequencing had brought it back to attention and was recently used in the new Roadmap Initiative in the Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) (Pei et al., 2009).

2.1.2.2 - Protein-encoding genes as molecular targets

2.1.2.2.1 - RNA polymerase encoding gene *rpoB*

RNA polymerase (RNAP) or 'DNA-dependent RNA polyermase' is a vital holoenzyme (apoenzyme + cofactor) responsible for RNA transcription and consequently, plays a key role in the gene expression process in all living species. (Borukhov et al., 2003; Adékambi et al., 2009). Bacterial RNAP is approximately 400 kDa in mass and its core complex is composed of five subunits; (α^{I}, α^{II}, β, β' and ω) forming a crab claw-like molecular structure (Zhang et al., 1999; Murakami, 2015). The β subunit, encoded by gene *rpoB* is the second largest subunit and contributes in the foremost catalytic activities of the RNAP (Jin and Gross, 1989). Numerous studies had reported the association of the *rpoB* gene in anti-microbial resistance to certain antibiotics such as rifampicin, a drug used in the treatment of *Mycobacterium tuberculosis* (Telenti et al., 1997; Fluit et al., 2001). The resistance has been attributed to a mutation occurring mainly between the 507 and 533 codons of the rifampin resistance-determining region (RRDR) of the *rpoB* gene (Smoskovi et al., 2001) and less commonly outside this zone (Siu et al., 2011). Complying to the benchmark for phylogenetic markers (Cruickshank, 2002; Zeigler et al., 2003; Patwardhan et al., 2014); present as a single-copy and ubiquitous gene with significant sequence diversity within closely related species, the RNA polymerase beta-subunit encoding gene *rpoB* has been selected as a competent phylogenetic marker to rectify the limitations in the 16S rRNA arising from the existence of multiple operons within a single genome (Drancourt and Raoult, 2002; Da Mota et al., 2004; Walsh et al., 2004). In various microbial taxonomic studies gene *rpoB* proved to be a more favorable and discriminative tool than the 16S rRNA. (Dahllöf et al., 2000; Case et al., 2007). In addition, it has been profoundly applied in molecular identification of aquatic and soil bacteria (Peixoto et al., 2002; Taylor et al., 2004), α-proteobacteria (Taillardat-Bisch et al., 2003), enteric bacteria (Mollet et al., 1997) and as a molecular target in the Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis for monitoring lactic acid bacteria (LAB) involved in food fermentation (Rantsiou et al., 2004). Furthermore, the RpoB protein, relatively 1.100 amino acids in size, was used in phylogenetic studies of Gram-positive and Gram-negative bacteria (Morse et al., 2002) and among Archeae (Matte-Tailliez et al., 2002). Moreover, based on a pair of universal oligonucleotide primer presented by Khamis et al. (2004), the gene *rpoB* was effectively used by Ülbegi (2010) in the delineation of the various species of genera *Arcanobacterium* and *Trueperella* and additionally, in the identification of two *A. (T.) pyogenes* strains isolated form

a bearded dragon and a gecko. Meanwhile, gene *rpoB* was used in lieu of the *v3* region of the SSU rRNA gene for the investigation of bacterial pathogens in dairy products (Deperrois-Lafarge and Meheut, 2012) and pertaining to its hyper variable region, was also used in restriction fragment length polymorphism (PCR-RFLP) technique for the discrimination between *C. pseudotuberculosis* and *A. (T.) pyogenes* (Pavan et al., 2012). In more recent studies, gene *rpoB* was engaged in collaboration with other target genes in genotypic characterization of *Arcanobacterium pluranimalium* isolated from milk samples and *A. hippocleae* isolated from the uterus of a healthy mare, respectively (Wickhorst et al., 2016; 2017b).

2.1.2.2.2 - Superoxide dismutase A encoding gene *sodA*

Superoxide dismutases (SODs), also known as the 'first line of antioxidant defense', are a group of broadly conserved catalyzing enzymes that are responsible for the detoxification of reactive oxygen species (ROS) against cellular damage. ROS are essential and non-essential oxygen species, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) resulting from partial oxygen reduction. Excessive formation of ROS is believed to be disease-associated and leads to a status of 'oxidative stress', causing cellular and DNA impairment (Thirach et al., 2007; Ray et al., 2012).

SODs could be categorized into copper-/zinc-containing SOD (Cu/ZnSOD) (McCord and Fridovich, 1969), manganese-containing SOD (MnSOD) (Keele et al., 1970), iron-containing SOD (FeSOD) (Yost and Fridovich, 1973) and nickel-containing SOD (NiSOD) (Youn et al., 1996). They act by transforming the superoxide free radical (O^-) into hydrogen peroxide and oxygen. The former is further broken down into water by catalase or peroxidase enzymes (Merkamm et al., 2001; Akaza et al., 2006).

The managanese-containing SOD (MnSOD) encoding gene *sodA* was proven to be of great benefit to microbial taxonomy of closely-related species. In consequence, it has been used for identification and differentiation of *Mycobacterium* species (Zolg and Philippi-Schulz, 1994), *Enterococcus* species (Poyart et al., 2000), *Streptococcus* species (Poyart et al., 1998) and species of genus *Staphylococcus* (Poyart et al., 2001). Furthermore, the amplification and sequencing of gene *sodA* for identification of various species of genera *Arcanobacterium* and *Trueperella* could successfully be achieved by means of the set of primer designed by Zolg and Philippi-Schulz (1994) and Ülbegi (2010).

2.1.2.2.3 - Genes encoding moonlighting proteins

Moonlighting proteins are described as a group of proteins undergoing diverse and independent functions that are irrelevant to each other, in such a way that restricting one function should have no impact on the other functions and contrariwise. (Huberts and van der Klei, 2010). This phenomenon was first reported by Piatigorsky and Wistow (1989) amid their study on crystallin enzymes present in the eye lens of vertebrates. In comparing these crystallins to prominent metabolic enzymes, such as argininosuccinate lyase (Piatigorsky, 1998) and lactate dehydrogenase (Hendriks et al., 1988), they displayed resemblance in structure but variation in functions. In consequence, this phenomenon was defined as 'gene sharing' (Piatigorsky et al., 1988). However, in context with its 'holding multiple jobs' metaphor these proteins became more prominently known as 'moonlighting proteins'. (Jeffery, 1999). Standard models of moonlighting proteins include DNA/RNA combining enzymes forming transcription cofactors (Commichau and Stulke, 2008), other proteins such as receptors, ribosomal proteins, chaperonins, elongation factor *Tu* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Pancholi and Chhatwal, 2003; Henderson and Martin, 2011). Since its first discovery, there have been more than hundreds of distinguished moonlighting proteins, compared to approximately 19,000 identified protein-encoding genes. However, this number seems to be ascending due to the growing tendency to study these proteins that were found to exist in all living organisms (Henderson and Martin, 2013). Moreover, to facilitate this pattern, online databases such as MoonProt Database (http://www.moonlightingproteins.org/) (Mani et al., 2015) and MultitaskProtDB (http://wallace.uab.es/multitask/aggregates) (Hernández et al., 2014) have been created which aim to aggregate all pertinent data about these pragmatic proteins which to date have constituted nearly 300 proteins (Jeffery, 2015; Gancedo et al., 2016). In addition, there have been considerable attempts to classify these proteins by clustering them into multiple groups based on their miscellaneous functions. This have served the inspection of their multiple vital roles such as protein-protein interaction, gene expression and phyletic lineage. In this regard, 33 newly defined moonlighting proteins have been reported in *E. coli* (Khan et al., 2014). Apparently, there may be still more moonlighting proteins to be discovered in the near future.

Numerous reports had described the main roles moonlighting proteins exhibit in prokaryotes which includes: (i) Adhesion, (ii) Plasminogen-binding and (iii) Modulation of host immune responses (Wang et al., 2014).

(i) Adhesion: it has been reported that specific pathogens exhibit cytoplasmic proteins on their cellular surface which are capable to bind to epithelial components (e.g. mucus) of the host or attach to the epithelial tissue. A common example of adhesive moonlighting proteins is the glyceraldehyde-3-phosphate dehydrogenase GAPDH which will interact with host components such as; binding of *Streptococcus pyogenes* with myosin and actin (Pancholi and Fischetti, 1992), *Mycoplasma pneumonia* with mucin (Alvarez et al., 2003) and enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* with fibrinogen and intestinal epithelial Caco-2 cells (Egea et al., 2007; Aguilera et al., 2009).

Other similar proteins which exhibit adhesion are enolase enzyme secreted by *Lactobacillus crispatus*, a commensal bacterium used in probiotics, which has been found to bind with mucin and fibronectin (Kinoshita et al., 2013).

(ii) Plasminogen-binding: plasminogen is a proenzyme belonging to the serine protease plasmin which is a vital component for fibrolysis (Lähteenmäki, et al., 2015). The binding of bacterial proteins such as GAPDH with the host plasminogen, thus activating its transform to plasmin, shall enhance tissue infringement and disintegration (Terao et al., 2006).

(iii) Modulation of the immune response of the host: several proteins such as EF-Tu secreted by *Lactobacillus johnsonii* and heath shock protein GroEL secreted by a diversity of Gram-positive bacteria are believed to interact with CD14 to promote the release of IL-8 by HT29 cells (Granato et al., 2004; Bergonzelli et al., 2006).

2.1.2.2.3.1 - Glyceraldehhyde 3-phosphate dehydrogenase encoding gene *gap*

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a highly conservative 37 kDa tetrameric housekeeping protein has been mainly considered for its vital glycolytic role inside the cellular cytoplasm of all three domains of life (Eukaryotes, Prokaryotes and Archaea). In this aspect, it acts as the sixth step catalyzer breaking down glucose for energy and carbon supply (Sirover, 1997).

D-glyceraldehyde 3-phosphate <==========> D-glycerate 1,3-biphosphate

+ Phosphate +NAD GAPDH + NADH

However, recent studies have underlined the extensive moonlighting effects GAPDH undertakes, relying on its structural heterogeneity and location within the living cell (Fillinger et al., 2000; Sirover, 2011). Its oligomeric structure and anomaly in specific amino acids determines GAPDH's bias towards either binding to NAD or NADP, thus demonstrating its

anabolic or catabolic carbon flux (Corbier et al., 1990; Clermont et al., 1993). Additionally, inside the nucleus, GAPDH protein serves as a binding activator for transcription, replication, maintenance and vesicular transport of nucleic acid polymers (Morgenegg et al. 1986; Singh et al., 1993, Meyer-Siegler et al., 1991; Zheng et al., 2003). Numerous studies have revealed the commitment of GAPDH in cellular reaction against oxidative stress (Dastoor and Dreyer, 2001; Hara et al., 2006; Aguilera et al., 2009) and its role in apoptosis (Nakajima et al., 2007) by acting as a nitric acid (NO) target, a major mediator in cellular apoptosis (Lipton et al., 1993; Benhar et al., 2005).

In prokaryotes, the multiple functions of GAPDH rely basically on its extracellular position and host binding secretions, which enable colonization and manipulation of the host cells (Pancholi and Chhatwal, 2003). It was initially discovered in Gram-positive bacterium, precisely in *Streptococcus (S.) pyogenes*, a β-hemolytic bacteria responsible for a wide range of infections in humans such as pharyngitis and skin disease (Lottenberg et al., 1992, Pancholi and Fischetti; 1992). Hence, GAPDH protein was found to act as a microbial surface binding receptor by interacting with various host proteins such as mycosin, actin and plasminogen, forming a protein complex that would degrade extracellular matrix proteins allowing microbial intrusion and colonization of host cells (Giménez et al., 2014). The host adhesion and virulence activity of GAPDH has been further investigated in a wide variety of microorganisms including *Streptococcus suis*, *Streptococcus pneumoniae* (Brassard et al., 2004; Bergmann et al., 2004; Ling et al., 2004), *Bacillus anthracis* (Matta et al., 2010) and *Mycoplasma pneumoniae* (Dumke et al., 2011). Meanwhile, the extracellular engagement of GAPDH in Gram-negative bacteria was merely revealed in the succeeding decade. Whereby, the extracellular binding of GAPDH in enterophathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* with plasminogen and fibrinogen at the Caco-2 cells, causing infection in humans, were displayed using western blotting and ELISA (Egea et al., 2007). Furthermore, it has been proposed by various authors that the interaction of GAPDH of probiotic bacteria, such as lactobacilli with intestinal mucin would facilitate adhesion and consequently, host intestinal colonization (Kinoshita et al., 2013; Martin et al., 2012). Moreover, the studies of pathogenic influence of GAPDH have been broadened to include its role in malaria infection (Daubenberger et al., 2003) and brucellosis (Fugier et al., 2003) and for the repair of cytotoxic DNA lesions in *E. coli* (Ferreira et al., 2015). In a more recent study, the peroxidase activity resulting from the interaction of GAPDH with heme protein and the protection role it plays as a chaperone has been elucidated (Huang et al., 2017).

For bacterial taxonomy, the application of glyceralehyde 3-phosphate dehydrogenase encoding gene *gap* has been mentioned in numerous studies. It has shown to be a useful taxonomic tool in the identification and genetic classification of *Staphylococcus* spp. by applying PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Yugeuros et al., 2000; 2001; Ghebremedhin et al., 2008) and sequencing of coagulase negative *Staphylococcus* spp. from human and bovine origin (Layer et al., 2006; Bal et al., 2010). Additionally, characterization of the *gap* operon in bacteria for bacterial identification has been demonstrated in *Lactobacillus (L.) plantarum* and *Lactobacillus (L.) sakei* by Naterstad and coauthors (2007). Moreover, the amplification and sequencing of gene *gap* using a newly designed set of primers could be successfully achieved for the reference strains of genera *Arcanobacterium* and *Trueperella* and for the identification and characterization of *Trueperella pyogenes* isolated from a brain abscess of an adult roebuck, *Arcanobacterium hippocoleae* collected from a uterine swab of an apparently healthy mare and *Actinomyces hyovaginalis* from a giraffe with suppurative arthritis, respectively (Wickhorst et al., 2017a, b, c).

2.1.2.2.3.2 - Elongation factor tu encoding gene *tuf*

Elongation factor thermo unstable (EF-Tu) is a monomeric G protein that weighs 43 kDa in *E. coli* and consists of 393 amino acids. It is one of the most highly conservative and abundant polypeptide in prokaryotes (Wittinghofer et al., 1983; Weijland et al., 1992). Its main enzymatic activity is to catalyze the binding and transportation of charged aa-tRNA (bound to an appropriate amino acid) to the A-site at the ribosome, thereby playing a crucial role in protein translation (Caldas et al., 1998).

The ribosome, consisting of a large 50S subunit and a 30S subunit in prokaryotes is considered the workshop for protein synthesis. Hence this process, also known as 'protein translation' is profiled in three major steps: 1) Initiation, 2) Elongation, and 3) Termination.

1) Initiation: The 30S ribosome is combined with the fMet-tRNA^{fMet} (*N*-Formylmethionyl-tRNA) and mRNA forming an initiation complex. Consequently, the 50S ribosome will associate with the 30S ribosome forming 70S ribosome. Thereupon, three binding sites are development on the associated ribosome; (A) aminocyl, (P) peptidyl and (E) exit.

2) Elongation: completion of the initiation step will onset the elongation process. EF-Tu will bind to the GTP (Guanidine-5'- triphosphate) and aminoacyl-tRNA (aa-tRNA) forming a EF-Tu • GTP • aa-tRNA ternary complex. The basic role of the EF-Tu is to protect the ester linkage formed between the tRNA and its annexed amino acid as it is transported to the ribosome

(Eargle et al., 2008). This EF-Tu • GTP • aa-tRNA will be delivered at the A-site of the ribosome where the anti-codon on tRNA will match to the codon of mRNA and bind to it through a hydrogen bond. This will lead to hydrolysis of GTP and the release of the EF-Tu (Becker and Kern; 1998; Curnow et al., 1998; Roy et al., 2007). The fMet will detach from the tRNA and couple the amino acid at the A-site through a peptide bond. In doing so, the freed tRNA will be moved to and then be released through the E-site, whereas, the newly connected fMet • aa-tRNA will substitute at the P-site, thus leaving the A-site for a newly delivered EF-Tu • GTP • aa-tRNA ternary complex to bind. Simultaneously, the ribosome will shift forward by one codon (translocation).

3) Termination: The operation continues and the polypeptide chain keeps on building until the process is terminated by the movement of a stop codon into the A-site (UAG/UAA/UGA). Correspondingly, the 70S ribosome is dissociated into 50S and 30S ribosomes, releasing the newly synthesized protein and the mRNA (Ramakrishnan, 2002).

It is noteworthy that protein synthesis is remarkably a concrete process, in which the decoding error ratio is estimated to be around 1 to every 10,000 codons (Kurland, 1992; Ibba, 2001).

Beside its crucial role in translation, EF-Tu has been found widely dispersed under the bacterial cell membrane in association with MreB, a protein similar in structure to actin that is known to be responsible for maintenance of bacterial cell shape (Mayer, 2003). In this regard, it has been observed that any deficiency in EF-Tu has a direct influence on the bacterial morphology (Mayer, 2006). Moreover, several reports have expressed the chaperone-like activities of the elongation factor EF-Tu in prokaryotic cells and in plants, where it catalyzes refolding of denatured proteins and combines to specific proteins, thus forming complexes that act as a safeguard in response to heat shock conditions (Kudlicki et al., 1997; Fu et al., 2012).

In addition, the role of EF-Tu encoding gene *tuf* in taxonomic studies has been widely expressed by numerous researches. It has been broadly implemented for the investigation and differentiation of various species including *Lactobacillus* species, *Bidifobacterium* species (Ventura et al., 2003) and *Yersinia* species (Isabel et al., 2008). Moreover, it has been reported in comparison to *gap* and MALDI-TOF MS analysis for the identification of *Staphylococcus* species (Bergeron et al., 2011) and as a molecular evaluation criterion for the investigation of *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* isolated from respiratory samples (Wessels et al., 2012). In 2016, Wickhorst et al. described oligonucleotide primer that allowed the partial amplification and sequencing of gene *tuf* for various reference strains of species of genera *Arcanobacterium* and *Trueperella* (Wickhorst et al., 2016). More recently, these primers have been used to investigate, a *T. pyogenes* strain isolated from a brain abscess

of an adult roebuck, an *A. hippocoleae* strain isolated from a mare and an *Actinomyces hyovaginalis* strain isolated from an adult giraffe, respectively (Wickhorst et al., 2017a, b, c).

2.1.2.2.3.3 - Heat shock protein or chaperonin CPN60 encoding gene *cpn60*

Chaperones, also known as heat shock proteins, are a widely existing family group consisting of molecular proteins that are key factors in the protein quality control (proteostasis) system, in terms of preventing erroneous unfolding, mitigating its consequence and ensuring the appropriate folding/re-assembling and functionality of proteins (Saibil, 2013).

Type 1 chaperonin monomers, also known as CPN60, GroEL or HSP60 are 60 KDa protein subunits that are ubiquitously distributed among prokaryotes, eukaryotes and numerous archaea. The first *groE* gene was discovered in *E. coli* where it has been observed that any impairment in this gene induced a prohibition of plating in various bacteriophage (Georgopoulos et al., 1972; Takano and Kakefuda, 1972). Furthermore, groEL protein in *E. coli* has revealed tangible amino acid isonomy to a chloroplastic protein responsible for ribulose biphosphate carboxylase assembly (Hemmingsen et al., 1988), henceforth, elucidating its role in folding/unfolding of proteins in all living organisms. Recently, there have been proofing evidence of the multifunctional characteristics of chaperonins further to their role in maintaining protein folding/unfolding stability (Lund et al., 2009). Hence, they were found to exhibit lectin activities (Benkirane et al., 1992), operate as membrane stabilizers (Török et al., 1997), as neurotoxin (Yoshida et al., 2001), in addition to their various activities in bacterial pathogenesis (Henderson et al., 2006).

By virtue of its ubiquity in nature, chaperonin encoding gene *cpn60* has been highly favored as a molecular taxonomic tool in phylogenetic researches (Hill et al., 2004). Consequently, a set of universal primer, corresponding to the nucleotides 274-828 of *E. coli cpn60* gene sequence, were proficiently designed for the amplification of the 549-567 bp region of gene *cpn60* in a diversity of microorganisms (Hill et al., 2004). Moreover, observing a sequence variation on species-specific basis had indicated the use of gene *cpn60* as a collateral target for the investigation of various bacterial species including *Staphylococcus* spp. (Goh et al., 1996), *Streptotoccus* spp. (Alber et al., 2004), *Lactobacilus* spp. (Blaiotta et al., 2008) and *Aeromonas* spp. (Miñana-Galbis et al., 2009).

In addition, a chaperonin database cpnDB had been established (http://www.cpndb.ca/) which congregates a huge data of cpn60 sequences including those for prokaryotes, eukaryotes and archaea (Hill et al., 2004). Furthermore, Hijazin (2012) had successfully designed a set of

oligonucleotide primers for the amplification and sequencing of various species of genera *Arcanobacterium* and *Trueperella* using the sequence of chaperonin encoding gene *cpn60* of *T. pyogenes* obtained from GenBank. Hence, the latter sequence has also been used as a target for the development of a loop-mediated isothermal amplification (LAMP) assay, allowing the molecular identification of *T. pyogenes* strains from various origins (Abdulmawjood et al., 2016).

2.1.2.3 - DNA base (G + C) content

The genomic mol% G + C content has been a prominent taxonomic tool considerably used for the characterization and identification of organisms to the species level. It is described as the ratio of cytosines and guanines against the whole sum of genomic nucleotides (Rosselló-Móra and Amann, 2001; Tindall et al., 2010; Mesbah et al., 2011). Whereas previously, the G + C content has been roughly estimated by applying indefinite conventional methods such as thermal denaturation (Marmur and Doty, 1962), buoyant density in CsCl (Schildkraut et al., 1962) and melting profiles (Owen et al., 1969) or more progressive methods including HPLC (Ko et al., 1977; Mesbah et al., 1989), at present it is more precisely calculated gaining advantage of the leading-edge genome sequencing technology (Meier-Kolthoff et al., 2014). Howbeit, when applied under standard conditions, the value difference between the conventional, moderate and modern methods falls in the range of 1.2 mol% and 2 mol% (Mesbah et al., 2011). According to Goodfellow and coauthors (1997), a genomic G + C content discrepancy over 10 mol % between two micro-organisms is a sufficient indication of representing two different species. Meanwhile, a 3 mol % (Mesbah et al., 2011) to 5 mol% (Rosselló-Móra and Amann 2011) G + C content remains a common threshold value for a within-species determination (Meier-Kolthoff et al., 2014). As reported by Yassin and coauthors (2011), the DNA G + C mol % of genus *Arcanobacterium* ranges between 52 – 57 mol % compared to 56 – 66 mol % among various species of genus *Trueperella*.

2.1.2.4 - DNA-DNA hybridization

DNA-DNA hybridization (DDH) or reassociation method is considered the golden standard for species identification. It was presented in the 1960s to assess genetic relatedness by genomic comparing of divergent microorganisms (Schildkraut et al., 1961) but was broadly approved for classification 20 years afterwards (Wayne et al., 1987; Sentausa and Fournier, 2013). At present, it has become mandatory for taxonomic frontrunner journals such as the

International Journal of Systematic and Evolutionary Microbiology (Caumette et al., 2015). It is presumed crucial particularly when strains share a 16S rRNA gene sequence identity of > 97 % (Tindall et al., 2010).

Referring to Róssello-Móra et al. (2011), several methods for DDH have been comprehensively reviewed by various authors (Goris et., 1998; Grimont, 1988; Grimont et al., 1980; Johnson, 1985, 1989; Owen and Pitcher, 1985; Stackebrandt and Liesack, 1993; Tjernberg et al., 1989; Rosselló-Móra, 2006) which all coincide in the measurement of the capacity and / or the constancy of the hybrid double-stranded DNA derived from a combination of denatured DNAs and might only defer in the kind of DNA label or the method of measurement. The hybridization process is implemented either in a free solution or by adhering the DNA to a concrete surface. In principle, the method of DNA-DNA hybridization allows to determine the genomic similarity between two species. Double-stranded fragments of DNA from two different species are denatured into single strands by heating to a specific temperature. The single strands from both species are intermixed at a gradually decreasing temperature allowing them to find their complementary partners and re-anneal forming a hybrid DNA. (Sikorski, 2010). Two different measurements can be admissible by this procedure; the relative binding ratio (RBR) or the increment in melting temperature (ΔT_m) and occasionally both. The RBR is based on the assessment of the double-stranded hybrid DNA for a couple of genomes compared to that of the reference DNA rendered under similar conditions. It is displayed in percentage, as the reference genome will typically hybridize to itself with a 100 % identity. In the labelling DNA technique, the binding ratio (BR) will measure the amount of double-stranded hybrid DNA compared to the overall sum of labelled DNA included in the process. The RBR is then assessed by matching the percentage of reassociation between heterologous and homologous reactions. On the contrary, the ΔT_m determines the melting temperature difference between homologous DNA compared to a hybrid DNA. The DNA melting temperature is proportional to its GC content (Schildkraut and Lifson, 1965; Turner, 1996). Hybrid DNA tend to melt prior to homologous DNA based on the fact that a lower base pairing will recommend a smaller thermal energy, thus displaying a high melting point difference between them. In concept, RBR contemplates the degree of double stranded DNA (Ullman and McCarthy, 1973; Stackebrandt and Goebel, 1994) in parallel to the sequence identity that the ΔT_m displays. Nevertheless, there remain a direct congruence between the values of both parameters with the RBR over 50 % corresponding to a ΔT_m under 4-5 ºC (Rosselló-Móra and Amann, 2001).

The RBR is more inimitably used in the description of species.
A DDH value of ≥ 70 % has been an imperative threshold for the delimitation of a species

group. However, the DDH cut-off used is not pertinent to all prokaryotic genera. Hence the 70 % cut-off value would not differentiate between various *Rickettsia* species (Drancourt and Raoult, 1994) as well as the two genera *Shigella flexneri* and *E. coli* which belong to the family *Enterobacteriacea*e, where it displays a cut-off level above the threshold level (Caumette et al., 2015). Another drawback of this technique is that it can merely present a surmised evaluation of average genetic analogy delimiting only associated species or subspecies. Additionally, establishing an increment database for this technique is not possible (Schleifer, 2009) and its protocols are considerably laborious and intricate and need to be implemented under extremely standardized environment (Moore et al., 2010; Rosselló-Móra et al., 2011; Sarethy et al., 2014). For these reasons and more, there has been a suggestion to replace this technique with more precise methods (Stackebrandt et al., 2002) such as the multilocus sequence analysis (MLSA) (Gevers et al., 2005) and the average nucleotide identity (ANI) (Konstantinidis and Tiedje, 2005).

2.1.2.5 - Restricted Fragment Length Polymorphosim (RFLP)

This technique, described by Saiki et al. (1985), termed sometimes as cleaved amplified polymorphic sequence (CAPS), is one of the earliest prominent genotying methods for the diagnosis of sickle cell anemia. The concept beyond this technique is to initially amplify a DNA fragment followed by the application of restriction endonuclease enzymes (Rasmussen, 2012). These enzymes will recognize and cleave specific locations causing an endonuclease digestion of DNA samples and leading to the production of various sized restricted DNA fragments. The resulting fragments are separated according to their length with polyacrylamide or agarose gel electrophoresis. Latterly, capillary and microchannel electrophoresis with a considerably higher quality output had been introduced (Sinville and Soper, 2007; Stellwagen and Stellwagen, 2009; Rasmussen, 2012).

Moreover, by encountering the polymorphism in nucleic acids the technique had served well in the study and detection of genetic and molecular diversity among living organisms (Ota et al., 2007). It has been desirably used for the identification and delimitation of game birds, closely related poultry species, game animals like roe and red deer and in some tamed ruminants such as mouflons (Fajardo et al., 2006; Saini et al., 2007; Fajardo et al., 2009; Rojas et al., 2009). Furthermore, the RFLP analysis of the 16S rDNA was first described by Carlotti and Funke in 1994 as a convenient tool for the identification and characterization of microorganisms pertaining to their phyletic relevance. It has been additionally reported by

Navarro et al. (1992) and Jensen et al. (1993) that RFLP analysis of internally transcribed spacer (ITS) could be a desirable tool for the delimitation among diverse prokaryotic species. Likewise RFLP of the 16S rRNA and 16S-23S rDNA were used for the analysis of acetic acid bacteria (Ruiz et al., 2000) and bacteria of genera *Campylobacter*, *Helicobacter* and *Arcobacter* (Marshal et al., 1999; Raut and Kapadnis, 2007).

The technique has many benefits as it is simple, sensitive and needs no commitment of advanced applications or capacious staff training. However, its drawback lies in being tedious, its requisition of precise restriction enzymes that might be expensive and its incongruity for high-throughput analysis.

Several versatile web-based search engines contribute in the RFLP primer design such as the http://cedar.genetics.soton.ac.uk/public_html/primer (Ke, et al., 2002) and http://bioinfo.bsd.uchicago.edu/SNP_cutter.htm (Zhang et al., 2005) and a specific website for selecting desired restriction enzymes via the restriction enzyme database (REBASE) (Roberts et al., 2007) at http://rebase.neb.com.

In preference, there exist various manual primer design software that could be found either commercial (e.g. Gentyx; DNASIS Pro) or costless (http://frodo.wi.mit.edu.nl/fgg/kgen/primer/SNP_Primers.html) (Van Baren et al., 2004; Ota et al., 2007).

2.1.2.6 - Random amplified polymorphic DNA (RAPD)

RAPD represents a method of polymerase chain reaction originally described by Williams et al. (1990) which became a prevalent molecular marker implemented in miscellaneous phyletic researches. It relies on the application of solitary, short and arbitrary oligonucleotide primer (between 8 to 12 nucleotide) and thus, does not require an anterior insight of the DNA sequence. The arbitrary primers will anchor to various loci on the DNA template and end up annealing and amplifying random chunks of the template if positioned in an admissible amplitude to one another. The amplified products are separated using agarose gels electrophoresis with concentration between 1.5 – 2.0 % and are detected by staining with an appropriate fluorescent nucleic acid dye. The resulting unique molecular patterns (polymorphisms) also known as RAPD markers have been broadly applied for genetic or linkage mapping, animal and plant breeding purposes, genetic structure of population, hybrid purity and DNA fingerprinting (Williams et al., 1990). The procedure is simple, fast and adequate as it does not require any DNA probes or sequence information and efficient with a

low DNA input and high amplification output. Likewise, arbitrary primers are cheap and needs no efforts in their design. On the contrary, however, RAPD results are not easy to analyze and recommend a mindful control of DNA quality and concentration together with the thermocycling conditions. Furthermore, any disaccord between primer and target might engender a weak or no band result (Kumar and Gurusubramanian, 2011).

Two new variants derived from the RAPD concept were later described; the DNA amplification fingerprinting (DAF) and the arbitrary primed polymerase chain reaction (AP-PCR) (Gwakisa, 2002). In the former, concise random primers about 5-8 base pairs in size are applied, hence, yielding a higher amount of amplicon reaction which are run on polyacrylamide gel and dyed by silver staining (Caetano-Anolles et al., 1991), whereas, the latter relies on applying relatively longer primers which are also separated on polyacrylamide gel after being tagged with a radioactive label (Welsh and McClelland, 1990).

2.1.2.7 - Amplified fragment length polymorphism (AFLP)

AFLP is a method of DNA fingerprinting first described by Vos et al. (1995) that was initially implemented for the characterization of plant genomes but later became broadly used in microbial typing (Koeleman et al., 1998; Gürtler et al., 2001). Principally, the procedure comprises few steps. First, a set of restriction enzymes; a rare 6-base cutter (EcoRI/5'-GAATTC-3') and a frequent 4-base cutter (MesI/5'-TTAA-3') are used to slice the genomic DNA into restriction fragments which are ligated at its restricted ends by DNA fragments termed as adapters. The ligated terminals act as specific primer binding sites during PCR amplification. A preselective PCR amplification step is carried out using specific adapter-compliment primers with an additional single selective nucleotide at its 3' end followed by a selective PCR amplification using primers with 3-5 additional nucleotides. Each additional selective nucleotide can scale down the number of DNA bands by 4 folds and equivalently reducing the number of amplicons generated. The yielded amplicons are then separated by gel electrophoresis producing different fingerprints for comparison and characterization. (Vos et al., 1995; Gibson et al., 1998). There are various types of AFLP techniques, nevertheless, the most common variants apply either a set of variant restriction enzymes with a set of primer for amplification or one single resctriction enzyme and a single primer. The procedure could be automated by fluorescence labelling of the PCR primers which could be detected using a DNA sequencer (Ranjbar et al., 2014). The AFLP technique maintains an immense discriminatory power and a high reproducible yield (Torpdahl et al., 2004; Ross et al., 2005; Giammanco et

al., 2007) and has been involved in diverse plant and microbial genetic studies such as genetic mapping, determination of genetic correlation among species, closely related species and cultivars and biogeographic patterns (Paun and Schönswetter, 2012).

2.1.2.8 - PCR ribotyping

Ribotyping is a molecular technique used to differentiate between bacterial species by comparing the variations in specific rRNA regions, mainly the region falling between the 16S and 23S rRNA specific restrictive enzymes such *Cla*I, *Eco*RI, or *Sal*I (Shanker et al., 2014). The fragmented DNA is separated by gel electrophoresis, conveyed to a nylon or nitrocellular membrane and hybridized to labelled rRNA probes. The emerging fingerprinting patterns are analyzed using a ribotyping automated system such as the 'Dupont Qualicon' Riboprinter (Austin and Pagotto, 2003).

2.1.2.9 - Repetitive sequence-based PCR (rep-PCR)

Repetitive element sequence-based PCR (rep-PCR) is a typing technique that targets short highly conserved repetitive sequences in distinct locations spread across the bacterial genome to differentiate between bacterial species. This method, first described by Versalovic and coauthors in 1991, could be achieved using oligonucleodtide complementary primers which break down bacterial DNA into small fragment that are separated by gel electrophoresis generating unique fingerprinting profiles. Based on the type of targeted sequences rep-PCR techniques could be classified into 3 groups (Versalovic et al., 1998).

1) REP-PCR: repetitive extragenic palindromic elements are nearly 33bp – 40 bp long that were found to exist in 500-1000 manifolds in every bacterial genome of *E. coli* and *Salmonella* species, thus, comprising nearly 1 % of the entire genome (Hiett and Seal, 2009). A successful microbial fingerprinting using REP-PCR has been applied for the identification of *Lactobacillus* species (Gevers et al., 2001), species differentiation within genus *Geobacillus* (Meintanis et al., 2007), for classification of various species of genus *Xanthomonas* (Tuang et al., 2009), and more recently, for studying *Streptococcus agalactiae* strains from fish origin in Malaysia (Amal et al., 2013).

2) ERIC-PCR: enterobacterial repetitive intergenic consensus, around 126 bp in length and aspires the transcribed regions on the bacterial genome. ERIC-PCR was used for evolutionary studies of *E. coli* (Wilson and Sharp, 2006), as an evalutation technique for various strains of

Corynebacterium pseudotuberculosis (Dorneles et al., 2014) and for molecular typing of pathogenic *E. coli* strains of the urinary tract (Ardakani and Ranjbar, 2016).

3) BOX-PCR: BOX elements are repetitive elements located on various conserved regions on the bacterial genome and comprise three different subunits; boxA (57bp), boxB (43bp) and boxC (50bp) as reported by Martin and coauthors (1992). BOX-PCR fingerprinting has been applied for *Streptococcus pneumonia*e (Belkum and Hermans, 2001), *Aeromonas species* (Tacão et al., 2005), *Pseudomonas syringae* and *Pseudomonas viridiflava* group (Marques et al., 2008), various species of genus *Streptococcus* (Lanoot et al., 2008) and clinical isolates of the species *Pseudomonas aeroginosa* (Wolska et al., 2011).

2.1.2.10 - Multilocus Sequence Typing (MLST)

MLST is a well informative and portable technique for the characterization of microorganisms by exploring their genetic correlation and detecting metamorphic variation among them. It was introduced by Maiden et al. in 1998 within an epidemiological scheme on the human pathogen *Neisseria meningitidis*. It basically involves PCR amplification and sequencing of various conserved and irrelevant genes of microorganisms under study (Chen et al., 2015). The resulting sequences for each loci are assigned random allelic numbers based on sequence variation between them, thus, composing an allelic profile commonly defined as the sequence type (ST) (Maiden, 2006).

Formerly, 11 genes were used for the MLST scheme with fragment sizes of about 470 bp but it was comparably found that using six genes was sufficient to display a similar resolution. The nomination of loci for the MLST scheme remains empirical based on the bacterial taxa and the quality of resolution needed, hence, protein-coding functional housekeeping genes remain the genes of choice as they are considered the most conservative in terms of genetic mutation. Currently numerous MLST schemes exist which are dedicated for the study of pathogenic bacterial and fungal species and are accessible online such as http://www/mlst.net and http://www.pubmlst.org. (Glaeser and Kämpfer, 2015). Nevertheless MLST is not only limited to epidemiological analysis but has also been applied for the identification of strains up to and below the species level and on a diversity of pathogenic microorganisms for evolutionary, pathogenic, ecological and microbiome studies (Byrnes et al., 2009; Meyer et al., 2009; Litvintseva and Mitchell, 2012). Literally, this technique was not meant to assess phylogenetic correlation but is fairly cluster dependent in terms of allelic profiles by displaying the analogy and evolution in sequence types and sequence complexes (Gevers et al., 2005).

2.1.2.11 - Whole genome sequencing

The genomic sequencing of *Haemophilus influenza* in 1995 by Sanger conventional sequencing had marked a milestone in molecular biology and a starting point of the genomic era (Fleischmann et al., 1995). Moreover, the development of the high-throughput or next generation sequencing (NGS) in 2005 transformed it into a much faster and inexpensive technique. This methodology availed from the shortgun sequencing approach, which was developed during the Human Genome Project (HGP) where large sizes of DNA could be successfully sequenced based on the sequence overlapping concept (Zhang et al., 2011). At present, the vast majority of genome projects rely on the shotgun sequencing in their genome sequencing process.

There are different next generation sequencing techniques, which fall into two catergories or platforms: (1) the high-end instruments, and (2) the bench-top instruments. The former is capable of producing high throughput and long reads and delivering huge number of prokaryotic genomes per run but is relatively overpriced and better fits in large centers and facilities. The most common machines adopting this system are the PacBio RS, HiSeq instruments, Genome Analyzer IIx, the SOLiD 5500 series and the 454 GS FLX+. Comparably, the latter produces a lower throughput and workflows at moderate prices and are more suitable for daily use genomic applications. This includes the 454 GS Junior, Ion PGM, Ion Proton and the MiSeq. Hence, the data produced by each platform comes in various formats (Loman et al., 2012).

Notwithstanding the hasty progress in sequencing technologies and the concurrent development of bioinformatics tools, sequencing a genome remains a challenging and highly demanding technique in terms of funds, time and know-how. Radically, a draft genome must contain the entire nucleotide base sequence in the target species, whereas no standard sequence for a species exists due to individual genomic variation.

The process of whole genome analysis could be divided into 3 main phases: (1) amplification and sequencing, (2) alignment (assembling) and (3) annotation. (Duan et al., 2010; Pabinger et al., 2013; Edwards and Holt, 2013; Ekblom and Wolf, 2014).

Amplification and sequencing. The genomic DNA is extracted and purified for the preparation of a library and is validated for its quality and quantity to verify adequacy of good-quality DNA. Basically, a good-quality DNA incorporates diverse sets of DNA fragments compared to a high amount of duplicant fragments present in low-quality DNA. Consequently, repetitive duplicant fragments will be dramatically replicated during PCR amplification at the expense of

diverse fragments resulting in reduced sequence coverage. The quality of DNA fragments is scrutinized by qPRC or fluorometry.

Following this step, the DNA is sheared into smaller fragments between 100 bp and 1000 bp in length using either the mechanical (physical) or enzymatic (chemical) methods. The most commonly mechanical methods used are: (1) nebulization: which acts by sqeezing DNA through tiny holes into a mist-form state. It is low-priced but with high input loss and non-uniform fragment sizes and (2) ultrasonication: a hydrodynamic process of fragmenting by exposing DNA to short intervals of sonication. It is more costy but with minimal loss and allows regular fragment size production. Moreover, the enzymatic method provides a more progressive technique which needs lower DNA input and offers simpler and faster processing. It depends on a fragmentase enzyme which is a combination of a nuclease and endonuclease that produce random double-stranded DNA fragments which are ligated by the adaptors for further amplification. In both procedures, specific adaptors are attached to the terminals of the template which serve as primer-binding sites for the amplification process.

Next-generation sequencing systems could further be catergorized based on the sequencing technology they adopt. The two most common methods are:

Mate pair sequencing: DNA is fragmented into 2-5 kb segments and labeled dNTPs are added to its terminals. Fragments are circulated and fragmented into smaller pieces of 400-600 bp. Only fragments with labelled dNTPs are gathered and adapators ligated to its terminals for further sequencing.

Pair-end Sequencing: DNA is fragmented into 600-800 bp segments and pair-end adaptors are added to both terminals. After amplification, fragments are sequenced from both sides.

Currently there is a tendency to drift away from conventional Sanger sequencing and Roche 454 sequencing for shorter read techniques such as Illumina HiSeq, SOLiD, the more recent Pacific Bionsciences (PacBio), IonTorrent and Illumina Moleculo.

Alignment (Assembling). Ahead of this phase few refining steps need to be performed:

(1) Quality, long-range GC content, repeats and duplicated reads (also known as masking) need to be evaluated using programs like FastQC (http://www.bioinformatics.bahraham.ac.uk/projects/fastqc).

(2) Trimming of reads emerging from PCR duplications (low-quality base calls) by various programs and scripts such as ConDeTri described by Smeds and Künstner (2011).

(3) Correction of device errors and lapses by means of the k-mer count.

(4) Removing primer and vector sequences and impurities resulting from library preparation using plain scripts such as cutadapt (Martin, 2012) or read aligners like BWA (Li and Durbin, 2009).

After refining, the shortgun data need to be assembled by mapping through alignment to an existing reference genome (Nielsen et al., 2011) or '*de novo'* where no reference genome exists, thus, generating a set of aligned fragments known as contigs. However, this process requires a diversity of tools and algorithms which depend on numerous factors such as the quality, size, speed and accuracy of the reads.

For long-reads generated by the conventional Sanger sequencing, the Celera, Arachne and PCAP assemblers are a good choice (Batzoglou et al., 2002; Huang et al., 2003; Denisov et al., 2008). On the other hand, short-reads are assembled using the overlap-layout-consensus (OLC) system (Miller et al., 2010) adopted by assemblers such as Edena, SGA and FERMI (Hernandez et al., 2008; Simpson and Durbin, 2012; Li, 2012), the extension-based methods or De Bruijn (Eulerian) graph algorithms (Nagarajan and Pop, 2013). The extension-based system is competent but also highly susceptible to errors, repeats and polymporphism (e.g. SSAKE and JR-Assembler) (Warren et al., 2007; Chu et al., 2013). Meanwhile, De Bruijin graphs-based assemblers which depend on splitting the reads into k-mers to form the graph nodes, such as SOAPdenovo (Luo et al., 2012), ALLPATHS-LG (Gnerre et al., 2011), ABySS (Simpson et al., 2009) and Velvet (Zerbino and Birney, 2008) are considered the most efficient (Compeau et al., 2011).

Consequently, determining the appropriate assembling strategy remains a challenge. For this reason, numerous universial tools were designed for the evaluation and assessment of genomic assembly, such as Assemblathon 1 (Earl et al., 2011), GAGE (Salzberg et al., 2012), REAPR (Hunt et al., 2013) and Assemblathon 2 (Bradnam et al., 2013).

The last phase in assembly is scaffolding, where relevant contigs need to be oriented and placed in a linear order to form scaffolds or super-contigs. During this process, it is common for gaps to be formed between contigs and are filled with 'N's. However, not only the length of 'N's could be predicted, but they could also be removed using information from the previous paired-end or mate-pairing sequencing step by means of programs such as Gap-Closer (Li et al., 2010), iMAGE (Tsai et al., 2010) and GapFiller (Boetzer and Pirovano; 2012).

Furthermore, a post-assembly validation step need to be performed in which contigs of assembled draft genome are ordered versus an appropriate reference genome, probably the closest existing complete genome. This step can be done using specific command-line tools

like MUMmer (Kurtz et al., 2004) or the java-based Mauve (Rissman et al., 2009; Darling et al., 2010) which searches for matching contigs of draft and reference genomes.

Annotation. To retrieve the utmost of a genome sequence, genomic annotation is required to find and identify the gene structural and funcational information such as protein-coding sequene (CDS), GC content, genomic islands, regulatory and metabolic pathways. Worth noting that efficient annotation is proportional to a well assembled genome; with minor gaps and ~ 90 % coverage (Médigue and Moszer; 2007; Yandell and Ence; 2012).

The annotation of bacterial genome could be implemented using automated web-based, tools such as BASys (Bacterial Annotation System) (http://wishart.biology.ualberta.ca/basys) and RAST (Rapid Annotation using Subsystem Technology) (http://rast.nmpdr.org/), de novo-based like Prokka and DIYYA or reference genome-based such as with RATT and BG-7 (Van Domselaar et al., 2005; Overbeek et al., 2005; Aziz et al., 2008; Stewart et al., 2009; Otto et al., 2011; Pareja-Tobes et al., 2012).

2.1.2.12 - Average Nucleotide Identity (ANI)

ANI, developed by Konstantinidis and Tiedje (2005) is defined as the mean value of nucleotide sequence identity between orthologous genes of two compared genomes, given in percentage (Sentausa and Fournier, 2013). Since then, it has been considered a powerful genomic correlation tool with high competence in bacterial and archeal taxonomy (Kim et al., 2014). Furthermore, it has been comparably suggested that a 95 - 96 % ANI value would be equivalent to the 70 % threshold of DNA-DNA hybridization (DDH) for species delimitation (Konstantinidis et al., 2006; Goris et al., 2007; Richer and Rosselló-Móra, 2009). Among all DDH-like, computer-involved *in silico* genomic parameters such as Blast distance phylogeny (GBDP) (Henze et al., 2005) and the maximal unique matches index (MUMi) (Deloger et al., 2009) ANI has been reported as the most broadly used technique and was proposed the 'next generation gold standard' for species delimitation, in replacement of DDH (Chan et al., 2012; Goris et al., 2007; Grim et al., 2013; Haley et al., 2010; Ritcher and Rosselló-Móra, 2009; Yi et al., 2012). Moreover, Ritcher and Rosselló-Móra (2009) reported that tangible ANI results could reputably be concluded with as little as ~ 20 % sequence coverage for each correlated genome. The ANI measurement approach by Konstantinidis and Tiedje (2005) was further upgraded with a more systematic mode by Goris et al. (2007) in which comparing orthologous genes was replaced by shredding the genome into 2070 bp segments which were individually compared to the segments of the other genome. Moreover, several web-based servers for

measuring the ANI of genomic pairs are available online such as: http://enve-omics.ce.gatech.edu/ani/ and http://jspecies.ribohost.com/jspeciesws/, which is connected to BLAST+ (ANIb), MUMmer (ANIm) and in correspondence with the indexes of tetranucleotide signatures (Tetra) (Richter et al., 2016).

2.2 Genus *Arcanobacterium*

2.2.1 History and taxonomy

Genus *Arcanobacterium* (*A.*), is a group of rod-shaped Gram-positive bacteria belonging to the family *Actinomycetaceae*. It was first described by Collins et al. in 1982b, having its name emanating from the Latin word ''*arcanus*" – meaning secretive or mysterious. The first species constituting to genus *Arcanobacterium* was *A. haemolyticum* (Collins et al. 1982b), formerly known as *Corynebacterium haemolyticum*, cultivated from wound infections of American soldiers (MacLean et al., 1946). Few years later, according to Ramos et al. (1997), *A. pyogenes*, *A. bernardiae*, *A. phocae* isolated from various pyogenic infections in animals and humans, blood and abscesses of human origin and from numerous tissues and body fluids of marine mammals, respectively, were being classified into this genus. In the succeeding decennium, genus *Arcanobacterium* was supervened by *A. pluranimalium* isolated from a porpoise and a deer (Lawson et al., 2001), *A. hippocoleae* withdrawn from the vaginal discharge of a mare (Hoyles et al., 2002), *A. bialowiezense* and *A. bonasi* isolated from prepuce of European bison bulls, (Lehnen et al., 2006) and *A. abortisuis* revealed from a placenta of a sow having miscarriage (Azuma et al., 2009).

Nonetheless, in the year 2011, according to a proposal by Yassin et al., and proceeding from comparative chemotaxonomic and phyletic considerations, this genus was in need for a taxonomic reclassification. Thereby, genus *Arcanobacterium* was reclassified to predicate *A. haemolyticum*, *A. phocae*, *A. pluranimalium*, *A. hippocleae* with *A. pyogenes*, *A. bialowiezense*, *A. bonasi* and *A. abortisuis* being incorporated into the newly described genus *Trueperella* (*T.*). (Yassin et al., 2011).

The present classification of genus *Arcanobacterium* acquired from the taxonomic portal of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/guide/taxonomy) and the prokaryotic nomenclature of the German Collection of Microorganisms and Cell Cultures https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date/prokaryotic-nomenclature-up-to-date.html) are disclosed in **Table 2**.

Table 2: Present classification of the family *Actinomycetaceae*, genera *Arcanobacterium* and *Trueperella* referring to the National Center for Biotechnology Information and the German Collection of Microorganisms and Cell Cultures.

Phylum	*Actinobacteria*
Class	*Actinobacteria*
Subclass	*Actinobacteridae*
Order	*Actinomycetales*
Suborder	*Actinomycineae*
Family	***Actinomycetaceae***
Genus	***Arcanobacterium***
Species	*Arcanobacterium haemolyticum*
Species	*Arcanobacterium hippocoleae*
Species	*Arcanobacterium phocae*
Species	*Arcanobacterium pluranimalium*
Species	*Arcanobacterium canis* (present study)
Species	*Arcanobacterium phocisimile* (present study)
Species	*Arcanobacterium pinnipediorum* (present study)
Species	*Arcanobacterium wilhelmae* (present study)
Species	*Arcanobacterium urinimassiliense*
Genus	***Trueperella***
Species	*Trueperella pyogenes*
Species	*Trueperella abortisuis*
Species	*Trueperella bernardiae*
Species	*Truperella bialowiezensis*
Species	*Trueperella bonasi*

2.2.2. - Growth conditions and morphology

Genus *Arcanobacterium* comprises a group of microaerobic, Gram-positive, non-acid fast rod-shaped bacteria (Collins et al., 1982b). They are neither motile nor sporulating bacteria, are pleomorphic in shape, appearing either in rod, coccoid, T or V shapes and measure approximately 0.3-0.8 x 1.0-5.0 µm in diameter, in newly grown colonies (Holt et al., 1994; Hirsh and Biberstein, 2004; Moore et al., 2010; Yassin et al., 2011). Bacteria of genus *Arcanobacterium* are microaerobic or facultative anaerobic that are amicably grown under a CO_2 enriched climate, particularly in a candle jar. They are best cultivated on 5 % blood agar incubated under an optimal temperature of 37 ºC for 48 h, displaying a varying zone of incomplete to complete hemolysis. However, they are incapable of tolerating exposure to 60 ºC for 15 min (Collins et al., 1982b).

2.2.3 - Species of genus *Arcanobacterium*

2.2.3.1 - *A. haemolyticum*

A. haemolyticum had acquired its name from the latin word for blood '*haema*' and '*lyticu*s' meaning losable or dissolvable, indicating to the well-known hemolytic characteristic of this species (Collins et al., 1982b). Since its first description by MacLean and coauthors in 1946, as Gram-positive strains recovered from wound infections of American soldiers, *A. haemolyticum* encoutered numerous controversial taxonomical classifications, where it was initially affiliated to genus *Corynebacterium* together with *Corynebacterium pyogenes* and *Corynebacterium bovis* (MacLean et al., 1946). Nevertheless, this classification has been debated by various authors (Barksdale et al., 1957; Barksdale, 1970; Jones, 1975; Minnikin et al., 1978; Schofield and Schaal, 1981; Collins et al., 1982a). Whereas, owing to the findings of Cummins and Harris (1956) and Barksdale et al. (1957) that *C. haemolyticum* and *C. pyogenes* have common cell wall composition grounds and pertain to genus *Streptococcus*, further phenotypical investigations by Barksdale et al. (1957) lead to the suggestion that *C. haemolyticum* is merely a mutation of *C. pyogenes*. Meanwhile, according to various phenotypic and genotypic studies, *C. haemolyticum* and *C. pyogenes* were in need for further taxonomic review and it was proposed that *C. pyogenes* should be assigned to genus *Actinomyces* as *Actinomyces pyogenes* (Schofield and Schaal 1981; Collins et al. 1982a). On their further study, Collins et al. (1982b) had redesignated *C. haemolyticum* to *A. haemolyticum*, to become the earliest member of the novel genus *Arcanobacterium*.

A. haemolyticum has been specified as facultative anaerobic, Gram-positive, asporogenous, non-motile and non-acid fast, catalase-negative bacterium that prevail in various bacillary forms (Collins et al., 1982b; Lämmler and Hartwigk, 1995). Apparently, young colonies measured around 0.75 mm in diameter after 24 h of culture on blood agar under an optimal temperature of 37 ºC, and developed to approximately 1.5-2.5 mm after 48 h, forming a complete zone of hemolysis on sheep blood agar (Collins et al., 1982b; Lämmler and Hartwigk, 1995). *A. haemolyticum* grown on rabbit or human blood agar will display enhanced hemolysis compared to sheep blood agar (Lämmler and Blobel, 1988; Cummings et al., 1993; Ülbegi-Mohyla et al., 2009).

In 1964, Fraser et al. outlined the antagonistic interaction of *A. haemolyticum* with staphylococcal β-hemolysin causing a zone of inhibition, displayed as an 'arrow head' shaped-hemolysis and termed 'reverse CAMP reaction'. This observation was later postulated by Souckova and Soucek in 1972 as a reaction occurring between *A. haemolyticum* secreted phospholipase (PLD) and β-lysin of staphylococcal group origin. The reverse CAMP reaction of *A. haemolyticum* has additionally been approved by various authors (Lämmler and Blobel, 1988; Lämmler, 1994; Linder, 1997; Almuzara et al., 2002; Tan et al., 2006; Ülbegi-Mohyla et al., 2009; Hassan et al., 2009; Hijazin et al., 2010).

As mentioned in numerous studies, *A. haemolyticum* showed antibiotic susceptibility to penicillin, gentamicin, rifampin, ciprofloxacin, cephalosporins, clindamycin, macrolides and vancomycin, resistance to trimethoprim-sulfamethoxazole and partial resistance to tetracycline (Carlson et al., 1994, 1999; Almuzara et al., 2002; García-de-la-Fuente et al., 2012).

The chemotaxonomic findings of the cell wall components of reference strain of *A. haemolyticum* DSM 20595^{T} had revealed - congruent to the other species of genus *Arcanobacterium* - a peptidoglycan cell wall type A5α with variation L-Lys-L-Lys-D-Glu, comprehended with phospholipid types diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (Yassin et al., 2011). In addition, *A. haemolyticum* possessed consecutive species-specific fatty acid percentage values of MK-9[H_4] respiratory menaquinone (85 %), straight-chain of unsaturated acids $C_{18:1}$ *ω9c* (37 %) and saturated acids $C_{18:0}$ (24.7 %) and $C_{16:0}$ (22.5 %), upon cultivation on blood agar (Lehnen et al., 2006).

The DNA mol % G + C content of *A. haemotlyticum* was found to range between 50 and 52 mol % (Collins et al., 1982b).

2.2.3.1.1 - Importance of *A. haemolyticum* causing infections in humans and animals

A. haemolyticum is commonly known as a human pathogenic bacterium associated with pharyngitis and soft tissue infections (Banck and Nyman, 1986; Waagner, 1991; Esteban (Esteban *et al.* 1994; Alos *et al.* 1995; Minarik *et al.* 1997; Skov *et al.* 1998; Goyal *et al.* 2005; Parija *et al.* 2005; Tan *et al.* 2006; Vargas *et al.* 2006) et al., 1994). Nevertheless, it has been sporadically found to diffuse and invade other sites resulting in bacteremia and deep seated infections (Minarik et al., 1997; Skov et al., 1998; Tan et al., 2006). Similarly, *A. haemolyticum* has also been associated with cases of endocarditis (Alos et al., 1995), pyrothorax (Stacey and Bradlow, 2005; Parija et al., 2005), arthritis and osteomyelitis (Goyal et al., 2005) and from a brain abscess (Vargas et al., 2006). Furthermore, *A. haemolyticum* was isolated from semen of a patient with primary infertility and was also recovered either solely or as a co-infection from patients with Lemierre's Syndrome (Fernandez-Suarez et al., 2009; Lundblom et al., 2010; Lee et al., 2012; Ying-Qun et al., 2013). It has also been recovered from respiratory tract infections of 51 patients at an emergency unit of a hospital in north Spain (Garcia-de-la-Feunte et al., 2012). More recently, *A. haemolyticum* has been further investigated in chronic and diabetic foot ulcers (Imirzalioglu et al., 2014; Kang et al., 2016), in cases of orbital necrotizing fasciitis (Stone and Harshbarger, 2015) and fatal sepsis (Frikh et al., 2016).

The role of *A. haemolyticum* in animals was primarily reported by Richardson and Smith (1968), Roberts (1969) and Von Vounan and Drescher (1996) for isolates from the semen of a bull, lungs of sheep and goat brain, respectively. The etiological role of *A. haemolyticum* recovered from a periodontal infection of a rabbit had been illustrated by Tyrrell et al. in 2002. The same year, *A. haemolytcium* had been characterized from isolates originating from the lungs of piglets suffering from pneumonia in Yugoslavia (Suvajdžić et al., 2002). The first recorded cases of *A. haemolyticum* in horses was communicated by Hassan et al. 2009, whereby, seven strains recovered from infections of six horses with insufficient data over their mode of transmission or degree of pathogenicity, were characterized phenotypically and genotypically. In the following year, *A. haemolyticum* secluded from a bovine necrotic fasciitis has also been investigated (Bancroft-Hunt et al., 2010). A second characterization of *A. haemolyticum* from a horse - notably recovered from a postcastrational wound - was scrutinized by Hijazin et al. (2010). Hence, the investigation of *A. haemolyticum* isolated from postmortem findings of a female European badger (*Meles meles*) weighing 7.5 kg had marked the first detection of this species in a wildlife host (Wragg et al., 2011).

2.2.3.1.2 - *A. haemolyticum* genome sequence

In the year 2010, as a part of the Genomic Encyclopedia of Bacteria and Archaea project, the complete genome of *A. haemolyticum* type strain DSM 20595^{T} (=ATCC 9345 =NCTC 8452), comprising 1,986,154 bp, 1,821 coding genes and 16S rRNA genes became publicly available on the National Center for Biotechnology Information (NCBI) under Genbank accession number: CP002045, (Yasawong et al., 2010).

2.2.3.1.3 - Putative virulence factors of *A. haemolyticum*

2.2.3.1.3.1 - Arcanolysin

Arcanolysin (ALN) is considered a cholesterol-dependent cytolysin (CDC), a newly discovered hemolysis-causing factor secreted by *A. haemolyticum*. It is apparently present in all *A. haemolyticum* strains and weighs around 64 kDa and incorporates a signaling sequence, a putative PEST sequence and a variant undecapepetide at the domain 4 location, a considerable segment for the toxin functioning. According to Lucas (2009), based on the draft *A. haemolyticum* genome sequence, the arcanolysin encoding gene *aln* measures 1,764 bp, with approximately 46.5 mol % G + C. The ALN protein consists of 587 amino acids, 44 of which are protein-secretion signaling amino acids. A successful cloning of arcanolysin-encoding gene *aln* had revealed its toxinomic activities as a cholesterol-dependent cytolysin (CDC), causing hemolysis of erythrocytes and cytolysis of human, rabbit, porcine and equine cultured cells (Jost et al., 2011).

2.2.3.1.3.2 - Phospholipase D

Phospholipase D (PLD) is a ubiquitous enzyme existing in almost all organisms (Eukaryotes, Prokaryotes and Archaea). PLD belongs to the phospholipase family, which from its name, is responsible for cleavage of phospholipids; hydrolyzing phosphatidylcholine (PC) into phosphatidic acid (PA) and free choline (Exton, 2002; Kolesnikov et al., 2012). This process is part of the mammalian cells' physiological activity expressed in connection with sphingomyelin during cytoskeletal restructuring (Linder, 1997) and as a cellular mediator during molecular signaling and protein-protein communication with numerous collaborator (Gomez-Cambronero, 2014).

The secreted protein of *A. haemolyticum* interacting with the staphylococcal β-hemolysin to produce the reverse CAMP reaction was accredited to phospholipase D (Souckova and Soucek, 1972).

Cloning and sequencing of the gene encoding for phospholipase D (*pld*) in *A. haemolyticum* was achieved by Cuevas and Songer in 1993. The PLD protein was successfully originated and weighed around 31.5 kDa. Comparable studies revealed that gene *pld* of *A. haemolyticum* displayed an approximately 65 % sequence similarity to *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* as its closest phospholipase D producing relatives (Cuevas and Songer, 1993; McNamara et al., 1995; Lucas et al., 2010). Only few studies so far had chronicled the role PLD protein plays in bacterial pathogenicity. Meanwhile, the main activity of PLD protein is clearly presented as a catalyzing enzyme responsible for the dissociation of sphingomyelin, thus augmenting vascular permeability of the host and allowing bacterial protrusion into the blood stream. A well-reviewed pathogenic example of PLD as an exotoxin is *C. pseudotuberculosis*, in which the encoding gene *pld* is considered the premier virulence factor (Hodgson et al., 1999). Moreover, there have been speculations about the horizontal transmission of PLD encoding gene *pld* from mammals to *Pseudomonas aeruginosa* (Spencer and Brown, 2015). In *Yersinia pestis*, it was found that PLD has no pathogenic role, albeit, was crucial for the endurance of the carrier flea, required for toxic blood products degradation (Hinnebusch et al., 2002).

2.2.3.1.3.3 Neuraminidases

Sialic acids derive their name from the Greek word ‘sialon’ for ‘saliva’ being primarily discovered in salivary mucins (Varki and Schauer, 2009). They are less commonly known as neuramines and were early identified by Blix (1938) and Klenk (1941). They correspond to a forty 9-carbon acidic α-keto sugar family group of monosaccharides that are widely distributed among the 3 domains of life, and are considered one of the most significantly studied molecules. There are two ways bacteria fulfill their needs of sialic acid; they either develop sialic acid dependently by means of neuraminidase (sialidase) enzymes ‘de novo biosynthesis’ as in *Campylobacter jejuni* and *Neisseria meningitides* (Vimr and Lictensteiger, 2002) or else, neuraminidase-deprived bacteria like *Haemophilus influenza* endeavor for free salic acids expressed by salidase-producing bacteria surrounding their environment (Shakhnovich et al., 2002).

Sialidases or neuraminidases (*N*-acetylneuraminyl hydrolase; EC 3.2.1.18), as labeled by Gottschalk (1956), are a group of glycosyl hydrolase enzymes that are responsible for the cleavage of sialic acids of glycoprotein and carbohydrate origin, turning them into an easily digestible carbon source for bacteria (Rosenberg, 1995). These enzymes were initially discovered by Burnet and Stone in 1946 and had formerly been termed 'receptor-destroying enzymes' (RDE) (Burnet et al., 1947), referring to their first detected role in deactivating the influenza virus receptors present on human erythrocytes, resultantly evading from the viral agglutinating effect on blood cells (Ada and French, 1959). In general, neuraminidases are considered virulence factors that play various roles mainly in bacteria that occupy the mucus membranes (Jost et al., 2001). They act by reducing the density of mucus (Gottschalk, 1960), thus facilitating bacterial intrusion into submucosal tissue. Furthermore, these enzymes are well known in boosting adhesion and invasion as well augmenting exposure of host to pathogenic toxins (Galen et al., 1992). Neuraminidases are being also involved in biofilm formation and in pathogenic interactions associated with fibrosis, pneumonia and influenza (Soong et al., 2006). Moreover, as mentioned by numerous authors, neuraminidases display no more than 20 – 30 % amino acid identity and comprise two conserved motifs, the RIP/RLP motif (Arg-Ile/Leu-Pro), and the (Ser-x-Asp-x-Gly-x-Thr-Trp) which are present in 4-5 repeats in the enzyme (Roggentin et al., 1989; Gaskell et al., 1995; Crennell et al., 1996; Jost et al., 2001). Neuraminidases are exhibited by the entire *A. haemolyticu*m strains, whereas their genomes incorporate two neuraminidase genes, tagged as NanH and NanA. NanH weighs approximately 92.2 kDa and comparably shares a range of 23.2 – 26.0 % amino acid identity and 39.4 – 42.2 % sequence similarity to the equivalent bacterial neuraminidases: Ned of *Micromonospora viridifaciens*, NanH of *A. naeslundii* and NanH of *T. pyogenes* (Lucas, 2009). Remarkably, it was observed that NanA and NanH were not comparably close to each other than to neuraminidases of other species (Lucas, 2009).

2.2.3.1.3.4 - Collagen binding proteins

Host adhesion is considered a major phase through which bacteria fulfills their colonizing and invasive process (Pietrocola et al., 2007). The host extracellular matrix (ECM) consists basically of structural glycoproteins such as collagen, fibrinogen, laminin and elastin (Jost and Billington, 2005). Hence, ECM acts as a host binding target for diverse pathogenic bacteria, perceived by their microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Bodén and Flock, 1989; Foster and Höök, 1998). The crucial adhesive

character of ECM of various pathogens have been underlined in numerous researches such as the M3 protein in *Streptococcus pyogenes* (Dinkla et al., 2003), CbsA of *Lactobacillus crispatu*s (Antikainen et al., 2002), YadA of *Yersinia enterocolitica* (Emödy et al., 1989) and the most prominent CbpA of *T. pyogenes* (Esmay et al., 2003). Moreover, two collagen binding proteins for *A. haemolyticum* have been described; Cpa and CbpA (Lucas, 2009). Whereas, cpa shared 38.0 % sequence similarity to a collagen adhesive counterpart protein of *Streptococcus pogyenes*, CbpA displayed a closer correlation of 35.7 % amino acid identity and 56.2 % sequence similarity to an equivalent *Corynebacterium jeikeium* collagen binding protein and shared 40.2 % sequence similarity to the CbpA of *T. pyogenes* (Esmay et al., 2003; Lucas, 2009). Nevertheless, only few details are known about the role these collagen binding proteins and their encoding genes *cpa* and CbpA play in *A. haemolyticum*.

2.2.3.2 - *A. hippocoleae*

A. hippocoleae had been initially described by Hoyles et al. in 2002 for an *Arcanobacterium* strain isolated from the vaginal discharge of a mare suffering from vaginitis. *A. hippocoleae* was isolated together with *Corynebacterium* spp. and coagulase-negative staphylococci and was given its name pertaining to its isolation origin; '*hippos*' – the latin word for horse and *colea* meaning vagina, referring to its site of isolation. *A. hippocoleae* was described as a facultatively anaerobic, non-acidfast, non-motile, non-sporulating, rod-shaped, Gram-positive bacterium (Hoyles et al., 2002).

The 1492 bp 16S rDNA sequence of *A. hippocoleae* shared 94.8 % to 95.7 % sequence similarity to the formerly existing species of genus *Arcanobacterium*, with *A. phocae* as its closest relative. For the second time, *A. hippocleae* was isolated from the urine of a horse by Cai and coauthors (2003), whereby it was being successfully secluded in pure culture. A further isolation of *A. hippocoleae* was from the abdominal and pulmonary content of an American Quarterhorse foal corpse after a still birth. It has been additionally isolated from the mare's placenta with signs of placentitis (Bemis et al., 2008).

Referring to a phenotypic study of genus *Arcanobacterium* by Ülbegi et al. (2009), *A. hippocleae* cultivated on rabbit blood agar under microaerobic conditions (in a candle jar) presented as an enhanced zone of hemolysis in comparison to sheep blood agar. Furthermore, *A. hippocleae* revealed a positive CAMP-like hemolytic reaction with *S. aureus* and *A. haemolyticum* as indicator strains.

In 2012, the first evaluation of *A. hippocoleae* using MALDI-TOF MS together with various other species of genus *Arcanobacterium* and *Trueperella* was implemented by Hijazin and coauthors using the type strain *A. hippocoleae* DSM 15539^T (Hijazin et al., 2012b).
Meanwhile, the latest report on *A. hippocleae* was from a uterus swab of an apparently healthy mare where it has been characterized phenotypically by biochemical property analysis, MALDI-TOF MS analysis and genotypically by investigating various molecular target genes (Wickhorst et al., 2017b).

2.2.3.3 - *A. pluranimalium*

A. pluranimalium was first described by Lawson et al. (2001), deriving its name from the words '*pluris*' for many and '*animalium*' which means animals referring to its isolation from various animal origins. *A. pluranimalium* was incipiently secluded from the spleen of a dead harbor porpoise and lung of a dead sallow deer. Depending on the outcome of polyphasic taxonomic study, both isolates shared 100 % 16S rDNA sequence homology to one another followed by a range of 93.9 % - 96.5 % to the other four species of genus *Arcanobacterium*, with *A. phocae* as its closest relative (Lawson et al., 2001).
A. pluranimalium has been defined as facultatively anaerobic, Gram-positive, asperogenous, non-motile, non-acid fast bacterium which are slightly curved and slender rod in shape (Lawson et al., 2001). Referring to the phenotypic findings of Ülbegi-Mohyla et al. (2009), the type strain *A. pluranimalium* DSM 13483^T cultivated on rabbit blood agar under microaerobic conditions (in a candle jar) showed an enhanced zone of hemolysis compared to that on sheep blood agar. Additionally, *A. pluranimalium* possessed a positive CAMP-like hemolytic reaction with β-hemolytic *S. aureus*, *S. agalactiae* and *A. haemolyticum* as indicator strains. In 2010, Ülbegi-Mohyla investigated *A. pluranimalium* recovered in a mixed culture from a dog with pyoderma (Ülbegi et al., 2010a). *A. pluranimalium* strains were additionally isolated in the following year from various sites in sheep including abortion material, semen, abscesses, viscera, and from a milk sample of a cow suffering from mastitis (Foster and Hunt, 2011). The first identification of *A. pluranimalium* by MALDI-TOF MS analysis was achieved by Hijazin and coauthors, amid a provisional evaluation for all species of genera *Arcanboacterium* and *Trueperella* by this thereupon novel technique (Hijazin et al., 2012b). Moreover, a further investigation of an *A. pluranimalium* strain recovered from mastitis of a Holstein-Friesian cow in Switzerland was carried out by Moser et al. (2013). In 2016, 3 *A. pluranimalium* strains also recovered from bovine milk samples were identified by phenotypic methods, MALDI-TOF

MS, by various molecular targets and by newly developed loop-mediated isothermal amplification (LAMP) assay (Wickhorst et al., 2016). The pluranimaliumlysin encoding gene *pla* of *A. pluranimalium*, a potential virulence factor of these species, was submitted into NCBI GenBank under accession number HE653976.

2.2.3.4 - *A. phocae*

A. phocae was originally described by Ramos et al. in 1997, for strains recovered from the peritoneal fluid of a common seal. *A. phocae* is derived from '*Phoca vitulina*' the Latin word for 'common seals'. Despite of the distinctive respiratory infections and septicemic conditions associated with the isolation of *A. phocae* strains, its role in pathogenicity need to be elucidated. A post factum medical review was performed in 2003 on marine mammals marooned across California central coast (USA) between the years 1994 and 2000 (Johnson et al., 2003). In this study, 141 *A. phocae* isolates were investigated from various inflammatory lesions of living and dead California sea lions (*Zalophus californianus*), Pacific harbor seals (*Phoca vitulina richardii*), northern elephant seals (*Mirounga angustirostris*), southern sea otters (*Enhyra lutris nereis*) and necropsy findings of a common dolphin (*Delphinus delphis*) that died of pulmonary complications (*Enhydra lutris nereis*) (Johnson et al., 2003). Few years later, *A. phocae* strains were recovered from multiple pathogenic lesions of two California sea lions deserted along the southern cost of California (Giovannini, 2010).

Remarkably, the first detection of *A. phocae* in fur animals has been reported by Nordgren and coauthors during a retrospective case-control analysis performed in 2014. The study aimed to investigate multiple cases of pyoderma affecting fur animals which broke out in Finland in 2007 and spread across a wide region of Scandinavia, causing serious economic losses to fur industry. The primary fur animal species studied were the captive minks (*Neovison vison*), blue foxes (*Vulpes lagopus*) and raccoon dogs (*Nyctereutes procyonoides*). Noteworthy that the clinical manifestations were relatively different among these species. Whereas, the disease patterns were viewed as skin pyoderma on facial region and paws of minks, it was observed in the form of anorexia accompanied by purulent eye discharges in blue foxes and were only locally detected on the paws of dog raccoons. (Nordgren et al., 2014). Notwithstanding the isolation of *A. phocae* from all diseased animals together with other *Staphylococcus* and *Streptococcus* species, the pathogenic role of this species has yet to be confirmed. In 2015, Chalmers and coauthors investigated several pododermatitis cases in farmed minks which were hypothetically correlated to a cross infection caused by feeding minks on the flesh of infected

seals (Chalmers et al., 2015). Consequently, two years after their previous report, Nordgren and his group underwent two further research studies on the pyoderma outbreak in fur animals (Nordgren et al., 2016a; 2016b). The first study characterized a new epidemic necrotic pyoderma (FENP) referring to *A. phocae* as a main causative factor (Nordgren et al., 2016a), followed in the second by induction of experimental infection of Mink, in which the speculations of *A. phocae* as a predominant causative agent of fur animal epidemic necrotic pyoderma (FENP) were being sustained (Nordgren et al., 2016b). Concurrently, an empirical study was carried out on 10 healthy white colortype male minks by Hammer and coauthors (2016), whereby, the aetiology of FENP was attributed to an adjuvant effect between *A. phocae* and *Streptococcus halichoeri*. Very recently, a post-mortem investigation on various soft tissue specimens of harbor seals from North Sea of Schleswig-Holstein, Germany, collected between 1996 and 2014 had described the isolation of *A. phocae* together with *Staphylococcus delphini, Bordetella bronchiseptica, Brucella spp., Clostridium perfringens, E. coli, Ersipelothrix rhusiopathiae,* B-hemolytic streptocci and *S. aureus* (Siebert et al., 2017).

A. phocae had been described by Ramos et al. (1997) as facultative anaerobic, Gram-positive non-motile, asporogenous, non-acid fast short rod-shaped bacteria. The bacterium becomes fully mature after incubation on blood agar for 24 to 48 h at 37 ºC (Ramos et al., 1997, Johnson et al., 2003). Morever, *A. phocae* had been shown to display a reverse CAMP-reaction with staphylococcal β-hemolysin and a positive CAMP-like reaction with *Rhodococcus equi* (Johnson et al., 2003), *Streptococcus agalactiae* and *Psychrobacter phenylpyruvicus* as indicator strains (Johnson et al., 2003; Ülbegi-Mohyla et al., 2009).

A phocae has been investigated phenotypically (Ramos et al., 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Ülbegi, 2010), by proteomic mass spectrometry using MALDI-TOF MS analysis (Hijazin et al., 2012b; Wickhorst et al., 2016) and Fourier transform infrared spectrometry (Nagib et al., 2016), and genotypically by sequencing the 16S rRNA gene (Ramos et al., 1997; Johnson et al., 2003), 16S-23S rDNA intergenic spacer region (Hassan et al., 2008; Chalmers et al., 2015) and the protein encoding genes *rpoB* (Ülbegi-Mohyla et al., 2010b), *cpn60* (Hijazin, 2012), *sodA*, *tuf* and *gap* (Wickhorst et al., 2016).

The gene encoding phocaelysin (*phl*) of *A. phocae* had been previously described by Ülbegi (2009). Gene *phl* for *A. phocae* DSM 10002 and *A. phocae* DSM 10003 were entered into NCBI GenBank under accession numbers FN999907 and FN99908, respectively.

Moreover, the complete genome sequence of *A. phocae* DSM 10002^T has been submitted to NCBI GenBank by Varghese and Submissions (2016) under accession number LT629804.

2.2.3.5 - *A. urinimassiliense*

A. urinimassiliense, a very recently - during a culturomics study - described *Arcanobacterium* species, has been recovered from the urogenital tract of a nearly two-month old female infant suffering from rotavirus gastroenteritis (Diop et al., 2017). This newly described species derived its name from the Latin words '*urina*' for urine and '*Massilia*' for Marseille in France, referring to the source origin and place from which the strain has been isolated. *A. urinimessiliense* was defined as irregular, non-motile, rod shaped Gram-positive bacterium that was catalase and oxidase negative. The bacterium appeared on blood agar in small colonies that were beige in color and measure nearly 200 µm in diameter. They were around 400-600 nm long and 300-600 nm wide. (Diop et al., 2017).

The type strain Marsielle-P3248^{T} could not be detected by MALDI-TOF MS analysis but was rather determined by 16S rDNA sequencing, sharing 94.7 % sequence similarity and 3.95 % sequence divergence with the type strain *A. phocae* DSM 10002^{T} as its closest relative (Diop et al., 2017).

The genome sequences of type strain Marseille-P3248^{T} has been submitted to GenBank under accession number LT598574 and to the Collection de Souches de l'Unité des Rickettsies (CSUR) under accession numbers LT598574 and P3248, respectively.

Chapter 3 - Publications

Properties of an *Arcanobacterium haemolyticum* strain isolated from a donkey

Eigenschaften eines Arcanobacterium haemolyticum-Stamms, isoliert von einem Esel

Osama Sammra[1], Anna Balbutskaya[1], Samy Nagib[1], Jörg Alber[1], Christoph Lämmler[1*], Amir Abdulmawjood[2], Markus Timke[3], Markus Kostrzewa[3], Ellen Prenger-Berninghoff[4]

Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Schubertstr 81, 35392 Gießen, Germany[1]
Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany[2]
Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany[3]
Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurterstr. 85-91, 35392 Gießen, Germany[4]

Summary

The present study was designed to characterize phenotypically and genotypically an *Arcanobacterium haemolyticum* strain (*A. haemolyticum* P646) isolated from a purulent nasal discharge of a donkey. *A. haemolyticum* P646 showed, compared to sheep blood, an enhanced hemolytic reaction on rabbit blood agar, a synergistic CAMP-like reaction with *Streptococcus agalactiae* and *Rhodococcus equi* as indicator strains, a reverse CAMP reaction in the zone of *Staphylococcus aureus* β-hemolysin and the typical biochemical properties of this species. The species identity could be confirmed by MALDI-TOF MS analysis, by sequencing the 16S rDNA and glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* and by amplification of *A. haemolyticum* specific parts of 16S-23S rDNA intergenic spacer region and 23S rDNA. A. *haemolyticum* P646 and the reference strain *A. haemolyticum* DSM 20595 were further characterized by amplification of the putative virulence genes encoding arcanolysin, phospholipase D, hemolysin A, CAMP factor family protein, a collagen binding protein and two neuraminidases which were presentfor *A. haemolyticum* DSM 20595. *A. haemolyticum* P646 showed a comparable gene spectrum but was negative for the genes encoding collagen binding

protein and neuraminidase H. To our knowledge, the present study is the first phenotypic and genotypic characterization of an *A. haemolyticum* strain isolated from a donkey.

Keywords: *Arcanobacterium haemolyticum*, donkey, MALDI-TOF MS, 16S rDNA, *gap*, virulence genes

Zusammenfassung

In der vorliegenden Arbeit konnte der *Arcanobacterium haemolyticum*-Stamm *A. haemolyticum* P646, isoliert aus dem eitrigen Nasenausfluss eines Esels, phänotypisch und genotypisch charakterisiert werden. *A. haemolyticum* P646 zeigte auf Kaninchenblutagarplatten, im Vergleich zu Schafblutagarplatten, eine verstärkte Hämolyse, eine synergistische, CAMP-ähnliche Reaktion mit *Streptococcus agalactiae* und *Rhodococcus equi* als Indikatorstämmen, eine umgekehrte CAMP-Reaktion in der Zone des *Staphylococcus aureus* ß-Hämolysins und die typischen biochemischen Eigenschaften dieser Spezies. Die Spezieszuordnung erfolgte im Weiteren durch MALDI-TOF Massenspektrometrie, durch Sequenzierung der 16S rDNA und des Glyceraldehyd-3-Phosphat-Dehydrogenase kodierenden Gens *gap* und durch Amplifizierung eines *A. haemolyticum*-spezifischen-Teils der 16S-23S rDNA-Intergenic Spacer Region und der 23S rDNA. *A. haemolyticum* P646 und der Referenzstamm *A. haemolyticum* DSM 20595 wurden im Weiteren charakterisiert durch Amplifizierung mutmaßlicher Virulenzgene. Die Virulenzgene kodieren Arcanolysin, Phospholipase D, Hämolysin A, ein CAMP Factor Family Protein, ein Kollagen-bindendes Protein und zwei Neuraminidasen, die für *A. haemolyticum* DSM 20595 gefunden werden konnten. *A. haemolyticum* P646 zeigte ein vergleichbares Genspektrum, war aber negativ für die Gene die das Kollagen-bindende Protein und die Neuraminidase H kodieren. Nach vorliegendem Wissen ist dies die erste phänotypische und genotypische Charakterisierung eines *A. haemolyticum*-Stamms, isoliert von einem Esel.

Schlüsselwörter: *Arcanobacterium haemolyticum*, Esel, MALDI-TOF MS, 16S rDNA, *gap,* Virulenzgene

Introduction

A. haemolyticum is a well-known pathogen in humans causing pharyngitis (Carlson et al., 1995) and also systemic and deep-seated infections (Skov et al., 1998; Tan et al., 2006). The occurrence of *A. haemolyticum* (formerly *Corynebacterium haemolyticum*) in animal infections appears to be rare. It had been isolated from bovine semen (Richardson and Smith 1968), ovine lung tissue (Roberts 1969) and from a periodontal tissue of a rabbit (Tyrrell et al., 2002). More recently, the isolation of *A. haemolyticum* had also been reported from infections of horses (Hassan et al., 2009; Hijazin et al., 2010), from a necrotizing fasciitis of a bull (Bancroft-Hunt et al., 2010) and by post mortem examination from a lymph node of a badger with a retropharyngeal abscess (Wragg et al., 2011). For some of the isolated *A. haemolyticum* strains of animal origin the identification was confirmed by molecular studies, mainly by 16S rDNA sequencing (Hassan et al., 2009; Hijazin et al., 2010; Wragg et al., 2011). The aim of the present study was to identify and further characterize an *A. haemolyticum* strain isolated from a specimen of a donkey phenotypically and genotypically using various molecular targets.

Materials and Methods

A. haemolyticum P646 of the present study was isolated together with *Staphylococcus sciuri*, *Enterobacteriaceae*, *Streptococcus equi* subsp. *zooepidemicus*, *Actinobacillus equuli*, *Staphylococcus epidermidis*, *α-haemolytic streptococci, γ-haemolytic streptococci*, *Pantoea* spp., yeasts and molds from a nasal swab of a 17 year old male privately owned Poitou donkey (*Equus asinus*, Baudet du Poitou) with purulent rhinitis. The donkey, originaly coming from France, belongs to farm in Germany with several Poitou donkeys. The *A. haemolyticum* strain was characterized phenotypically, also including hemolytic properties on sheep and rabbit blood agar, CAMP-like activities, and the reverse CAMP reaction (Ülbegi-Mohyla et al., 2009; Hassan et al., 2009; Hijazin et al., 2010), and by MALDI-TOF MS analysis (Hijazin et al., 2012a,b,c). For molecular identification, the bacterial strain was investigated by amplification and sequencing of the 16S rDNA (Hassan et al., 2009) and glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap*, and, as described by Hijazin et al. (2010), by amplification of an *A. haemolyticum* specific region of 16S-23S rDNA intergenic spacer region (ISR) and 23S rDNA. The sequence of gene *gap* was obtained from the *A. haemolyticum* genome project (CP002045, accession no. ADH92610), the used oligonucleotide primers are given in Table. 1. Sequencing was performed

by Seqlab-Sequence Laboratories, Göttingen, Germany, alignment studies using DNASTAR Lasergene Version 8.0.2 (DNASTAR Inc., Madison, USA), Clustal W method. *A. haemolyticum* P646 was further characterized by PCR-mediated amplification of the genes encoding the potential virulence factors arcanolysin (ACV96715), phospholipase D (ADH92172), hemolysin A (ADH 92668), CAMP factor family protein (ADH92080), collagen binding protein (ADH93475), neuraminidase A (ADH91794) and neuraminidase H (ADH93004). The oligonucleotide primers were described previously (Hassan et al., 2009; Hijazin et al., 2010) or were newly designed using the sequences given by the *A. haemolyticum* genome sequencing project with the help of the NCBI primer design tool. The primer sequences and the PCR programs are shown in Table. 1. *A. haemolyticum* DSM 20595 and other reference strains of genus *Arcanobacterium* were used for control purposes (Hijazin et al., 2012b,c,; Hijazin et al., 2013).

Results and discussion

A. haemolyticum P646 produced a narrow zone of complete hemolysis on sheep blood agar and, compared to sheep blood, an enhanced hemolysis on rabbit blood agar, a synergistic CAMP-like reaction with *Streptococcus agalactiae* and *Rhodococcus equi* as indicator strains, a reverse CAMP reaction in the area of staphylococcal β-hemolysin and had the typical biochemical properties of this species (Tab. 2). The hemolytic properties on rabbit blood agar, the CAMP-like activities and the reverse CAMP reaction are known as typical characteristics of *A. haemolyticum*, also including *A. haemolyticum* of animal origin (Lämmler and Blobel, 1988; Hassan et al., 2009; Ülbegi-Mohyla et al., 2009; Hijazin et al., 2010). *A. haemolyticum* P646 showed a moderate liquefaction of Loeffler medium and no cross-reaction with streptococcal serogroup B or serogroup G-specific antisera (Tab. 2). A moderate liquefaction of Loeffler medium had also been observed for the previously studied *A. haemolyticum* strains from horses (Hassan et al., 2009; Hijazin et al., 2010). However, the extracellular substance causing the moderate serum liquefaction of the previously described *A. haemolyticum* from horses and *A. haemolyticum* P646 of the present study is not known. Liquefaction of Loeffler medium and a cross-reaction with streptococcal serogroup G-specific antisera are typical properites of *Trueperella pyogenes* (Bisping and Amtsberg, 1988; Lämmler and Hartwigk, 1995). A cross reaction of *A. haemolyticum* with streptococcal serogroup B-specific antisera, which could be observed in the

present study for *A. haemolyticum* DSM 20595, was described as common properties of *A. haemolyticum* isolated from human origin (García-de-la-Fuente et al., 2012).

As shown by numerous authors MALDI-TOF MS appears to be a powerful tool for species characterization of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011). Comparable to the previously conducted MALDI-TOF MS analysis of bacteria of genera *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012a,b,c), MALDI-TOF MS allowed the identification of *A. haemolyticum* P646 of the present study to the species level matching to *A. haemolyticum* DSM 20595 with a log (score) value of 2.20. The next relative among the reference strains of genus *Arcanobacterium* was *A. phocae* DSM 10002 with a low log (score) value of 0.97.

Sequencing 16S rDNA and gene *gap* of *A. haemolyticum* P646 showed a sequence similarity of ≥ 99.7 % and ≥ 99.9 % to 16S rDNA and gene *gap* of *A. haemolyticum* DSM 20595, respectively (Table 2). A dendrogram analysis is shown in Figure 1. Sequencing of gene *gap* had already been described for molecular identification of *Staphylococcus* spp. (Yugueros et al., 2000). In the present study this gene could be used as novel target gene for molecular identification of various species of genus *Arcanobacterium,* also including *A. haemolyticum*. The species identity of *A. haemolyticum* P646 could also be confirmed by previously described PCR-mediated amplification of a species-specific region of ISR and 23S rDNA (Tab. 2). As described previously (Hijazin et al., 2010) this species-specific PCR revealed no cross-reaction with various other species of genus *Arcanobacterium* indicating the usefulness of this molecular target for identification of *A. haemolyticum. A. haemolyticum* P646 and *A. haemolyticum* DSM 20595 could be further characterized by PCR-mediated amplification of the genes encoding the virulence factors arcanolysin and phospholipase D and the putative virulence factors hemolysin A, CAMP factor family protein and neurminidase A which appeared to be present in both strains. The potential virulence genes encoding collagen binding protein and neuraminidase H could only be found in *A. haemolyticum* DSM 20595 (Tab. 2). The pathogenic importance of the virulence factors arcanolysin and phospholipase D had been shown by Jost et al. (2011) and Lucas et al. (2010). A collagen binding protein and two different neuraminidase enzymes were already described as potential virluence factors of *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) (Jost et al., 2005) and more recently also as potential virulence factors of *A. haemolyticum* (Lucas, 2009). Further studies will give information about the distribution of these virulence genes among *A. haemolyticum* isolated from humans or animals and about the possible relation of these putative

virulence genes and clinical symptoms. However, the clinical importance of *A. haemolyticum* P646 of the present study, which was isolated together with several other potentially pathogenic bacterial species from the purulent nasal discharge of a Poitou donkey suffering from rhinitis, remains unclear. Besides the above mentioned studies about *A. haemolyticum* isolated from animal origin this is the third report about the isolation of *A. haemolyticum* from *Equidae* indicating that this bacterial species is also of some importance in veterinary medicine.

Conflict of interest

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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Address for correspondence:

Prof. Dr. Christoph Lämmler
Institute für Pharmakologie und Toxikologie, Justus-liebig-Universität Gießen
Schubert Str. 81, 35392 Gießen, Germany
Christoph.laemmler@vetmed.uni-giessen.de

Table 1: *Oligonucleotide primer sequences and PCR conditions of the target genes used in the present study*

Oligonucleotide primers	Sequence	Program*	Expected siz of PCR produ (bp)	References
1. 16S rDNA UNI-L 2. 16S rDNA UNI-R (amplification primer)	5'-AGAGTTGATCATGGCTCAG-3' 5'-GTGTGACGGGCGGTGTGTAC-3'	1	1,403	(Hassan et al., 2009)
3. 16S rDNA-533F 4. 16S rDNA-907R (Sequencing primer)	5'-GTGCCAGCMGCCGCGGTAA-3' 5'-CCGTCAATTCMTTTGAGTTT-3'	-	-	(CLSI, 2008)
5. Gap-F 6. Gap-R	5'-TCGAAGTTGTTGCAGTTAACGA-3' 5'-CCATTCGTTGTCGTACCAAG-3'	2	830	Present study
7. ISR-23S specific-F 8. ISR-23S specific R	5'-CCTAGCCTGGTGGTTGGGTAG-3' 5'-GTGCGGGTAACCAGAAATAACTCTG-3'	3	345	(Hijazin et al., 2010)
9. Arcanolysin-F 10. Arcanolysin-R	5'-TCCGAACAATCCTCACGAGC-3' 5'-GATCTCGCGCTCCTTAACCA-3'	4	754	Present study
11. pld-Phospholipase D F 12. pld-phospholipase D R	5'-ATGTACGACGATGAAGACGCG-3' 5'-GCTTCCTTGTCGTTGAGATTATTAGC-3'	5	528	(Hassan et al., 2009)
13. Hemolysin A F 14. Hemolysin A R	5'-GAGACGGTGCGATGGCCTGG-3' 5'-ACGTGCCTTGGATGCAGGCG-3'	6	434	Present study
15. Camp-F 16. Camp-R	5'-ACTTGCTTGACCGTGGAGCGTG-3' 5'-TCTCAGTAGTGTCCGCGCACG-3'	7	650	(Hijazin et al., 2010)
17. Collagen binding-F 18. Collagen binding-R	5'-CAGCCATTGTAGCGTTGGTG-3' 5'-CCCATCGCATCATTCGGGTA-3'	8	343	Present study
19. Neuraminidase A F 20. Neuraminidase A R	5'-AAGGCTGAATCTGAACGCGA-3' 5'-TCTGCCGTGATCTTGGTGTC-3'	9	689	Present study
21. Neuraminidase H F 22. Neuraminidase H R	5'-TGGAGAGCATCAAGCAGTTTC-3' 5'-TTCGACTATTTGCGTGTGACG-3'	8	329	Present study

*PCR Program

1: x1 (95°C, 600 sec), x30 (95°C, 30 sec, 58°C, 60 sec, 72°C, 60 sec), x1 (72°C, 420 sec).

2: x1 (94°C, 180 sec), x30 (94°C, 30 sec, 50°C, 40 sec, 72°C, 60 sec), x1 (72°C, 300 sec).

3: x1 (95°C, 240 sec), x30 (95°C, 8 sec, 66°C, 10 sec, 72°C, 10 sec), 1x (72°C, 420 sec).

4: x1 (95°C, 180 sec), x30 (95°C, 30 sec, 54°C, 30 sec, 72°C, 60 sec), 1x (72°C, 300 sec).

5: x1 (95°C, 600 sec), x30 (95°C, 30 sec, 60°C, 60 sec, 72°C, 60 sec), x1 (72°C, 420 sec).

6: 1x (94°C, 180 sec), 30x (94°C, 45 sec, 62°C, 30 sec, 72°C, 45 sec), 1x (72°C, 420 sec).

7: x1 (95°C, 240 sec), 30x (95°C, 45 sec, 55°C, 60 sec, 72°C, 60 sec), 1x (72°C, 420 sec).

8: 1x (94°C, 180 sec), 30x (94°C, 45 sec, 58°C, 30 sec, 72°C, 60 sec), 1x (72°C, 420 sec).

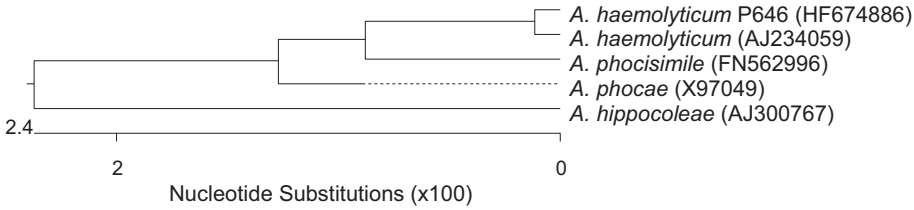
9: 1x (94°C, 180 sec), 30x (94°C, 45 sec, 54°C, 30 sec, 72°C, 60 sec), 1x (72°C, 420 sec).

Table 2: *Phenotypical and genotypical properties of A. haemolyticum P646 investigated in the present study and A. haemolyticum DSM 20595.*

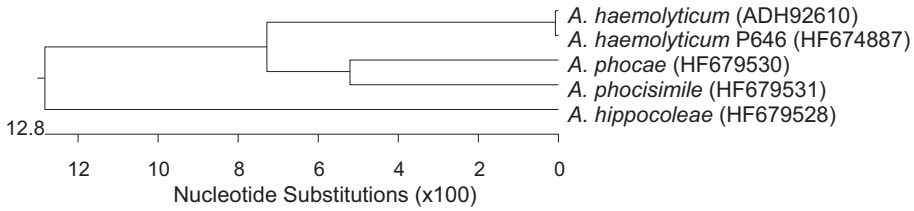
Phenotypic properties	*A. haemolyticum* P646	*A. haemolyticum* DSM 20595**
Hemolysis on sheep blood agar	+	+
Hemolysis on rabbit blood agar	++	++
CAMP-like reaction with:*		
Streptococcus agalactiae	+	+
Rhodococcus equi	+	+
Reverse CAMP reaction	+	+
Nitrate reduction	-[1]	-[1]
Pyrazinamidase	+[1]	+[1]
Pyrrolidonyl arylamidase	+[1]	-[1]
Alkaline phosphatase	+[1,2]	+[1,2]
β-Glucuronidase (β-GUR)	-[1,2,3]	-[1,2,3]
α-Galactosidase (α-GAL)	-[2]	-[2]
β-Galactosidase (β-GAL)	+[1,3]	+[1,3]
α-Glucosidase (α-GLU)	+[1,2,3]	+[1,2,3]
β-Glucosidase (β-GLU)	-[2]	-[2]
N-acetyl-β-Glucosaminidase (β-NAG)	+[1,3]	+[1,3]
Esculin (β-Glucosidase)	-[1]	-[1]
Urease	-[1]	-[1]
Gelatine	-[1]	-[1]
Fermantation of:		
Glucose	+[1]	+[1]
Ribose	+[1]	+[1]
Xylose	-[1]	-[1]
Mannitol	-[1]	-[1]
Maltose	+[1]	+[1]
Lactose	+[1]	+[1]
Saccharose	-[1]	-[1]
Glycogen	-[1]	-[1]
α-Mannosidase	+[2]	+[2]
Catalase	-	-
Serolysis on Loeffler agar	(+)	-
Caseinase	-	-
DNase	+	+
Starch hydrolysis	+	-
Cross-reaction with streptococcal serogroup B-specific antiserum	-	+
Cross-reaction with streptococcal serogroup G-specific antiserum	-	-

Molecular identification		
16S rDNA	+	+
gap	+	+
ISR-23S rDNA	+	+
Genes encoding potential virulence factors		
Arcanolysin	+	+
Phospholipase D	+	+
Hemolysin A	+	+
CAMP factor family protein	+	+
Collagen binding protein	-	+
Neuraminidase A	+	+
Neuraminidase H	-	+

The reactions are shown as follows:*= synergistic CAMP-like reaction with indicator strains; **= results mostly obtained from Hassan et al. (2009) and Hijzain et al. (2010); ++, enhanced positive reaction; +, positive reaction; (+), weak positive reaction; -, negative reaction 1 = Api-Coryne test system (Biomerieux, Nürtingen, Germany); 2 = tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); 3 = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).



(a)



(b)

Figure 1: *Dendrogram of sequences of the 16S rDNA (a) and gene gap (b) of A. haemolyticum P646 of the present study and of various reference strains of genus Arcanobacterium obtained from NCBI GenBank.*

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Phenotypic and genotypic characteristics of *Arcanobacterium haemolyticum* isolated from clinical samples in a Danish hospital

Osama Sammra · Alice Friis-Møller · Anna Balbutskaya · Muaz Hijazin · Samy Nagib · Jörg Alber · Christoph Lämmler · Amir Abdulmawjood · Markus Timke · Markus Kostrzewa · Ellen Prenger-Berninghoff

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Abstract Six *Arcanobacterium haemolyticum* strains isolated from six patients of two hospitals in Denmark were identified phenotypically, also including matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, and by genotypic methods. The latter were performed by sequencing 16S rDNA and glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap* and by amplification of an *A. haemolyticum* specific region of 16S–23S rDNA intergenic spacer region and 23S rDNA. The six *A. haemolyticum* strains were further investigated for the presence of seven potential virulence genes encoding arcanolysin, phospholipase D, hemolysin A, CAMP factor family protein, collagen binding protein, neuraminidase A and neuraminidase H which appeared to be present in two (seven virulence genes), two (six virulence genes) and two strains (four virulence genes), respectively. The phenotypic and genotypic properties described in the present study might help to reliably identify and further characterize *A. haemolyticum* isolated from human patients, a species which seems to be of increasing importance.

O. Sammra · A. Balbutskaya · M. Hijazin · S. Nagib · J. Alber · C. Lämmler (✉)
Institut für Pharmakologie und Toxikologie,
Justus-Liebig-Universität Gießen, Schubertstr 81, 35392 Gießen, Germany
e-mail: christoph.laemmler@vetmed.uni-giessen.de

A. Friis-Møller
Department of Clinical Microbiology, University Hospital,
Hvidovre, Kettegårds alle 30, 2650 Hvidovre, Denmark

A. Abdulmawjood
Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15,
30173 Hannover, Germany

M. Timke · M. Kostrzewa
Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany

E. Prenger-Berninghoff
Institut für Hygiene und Infektionskrankheiten der Tiere,
Justus-Liebig-Universität, Frankfurterstr. 85-91, 35392 Gießen, Germany

Genus *Arcanobacterium*, originally classified by Collins et al. (1982), consists of a group of facultatively anaerobic asporogenic Gram positive rods. According to Yassin et al. (2011), the genus was in need of a taxonomic revision. These authors proposed that genus *Arcanobacterium* should be split into two genera, with *A. haemolyticum*, *A. hippocoleae*, *A. phocae* and *A. pluranimalium* constituting genus *Arcanobacterium* and *A. pyogenes*, *A. abortisuis*, *A. bonasi, A. bernardiae*, *A. bialowiezense* being transferred to the new genus, *Trueperella* (Yassin et al. 2011). More recently, the novel species *A. canis* and *A. phocisimile* were described (Hijazin et al. 2012b, 2013). *A. haemolyticum* was first described as *Corynebacterium haemolyticum* in 1946 as a cause of pharyngitis and soft tissue infections in American soldiers (Maclean et al. 1946). A pharyngitis caused by *A. haemolyticum* resembles that caused by *Streptococcus pyogenes* and occurs mainly in adolescents and young adults (Banck and Nyman 1986; Miller et al. 1986; Balikci et al. 2011). More rarely, *A. haemolyticum* is responsible for invasive diseases which seem to occur more in old immune compromised patients (Brown et al. 2013). Case reports of systemic and deep seated infections caused by *A. haemolyticum* and a review of the literature were given by Skov et al. (1998), Tan et al. (2006) and Therriault et al. (2008). However, emphasizing the increasing importance of *A. haemolyticum* in clinical samples of human patients, García-de-la-Fuente et al. characterized in 2012, 56 *A. haemolyticum* isolated from respiratory infections of 51 patients which attended primary health care centres and

emergency units of an area in northern Spain. Comparable to these studies, the present investigation was designed to further characterize six *A. haemolyticum* strains isolated from clinical samples in Denmark.

The six *A. haemolyticum* strains were isolated from clinical samples of six patients attending two hospitals in Copenhagen, Denmark. Further data about the samples and the isolates are given in Table 1. For comparative purposes *A. haemolyticum* DSM 20595 and previously described reference strains of genus *Arcanobacterium* were included (Sammra et al. 2014). The six *A. haemolyticum* strains were investigated for hemolytic properties on sheep and rabbit blood agar, for CAMP-like reactions, for the reverse CAMP reaction, for biochemical properties and by MALDI-TOF MS analysis as described (Hijazin et al. 2012a; Sammra et al. 2014) and genotypically by amplification and sequencing of the 16S rDNA and the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap* (Hassan et al. 2009; Sammra et al. 2014). The strains were also identified by amplification of an *A. haemolyticum* specific region of ISR and 23S rDNA as described by Hijazin et al. (2010) and further characterized by amplification of the genes encoding the potential virulence factors arcanolysin, phospholipase D, hemolysin A, CAMP factor family protein, collagen binding protein, neuraminidase A and neuraminidase H. The primer sequences and PCR programs were described previously (Sammra et al. 2014). For amplification of the genes encoding collagen binding protein, neuraminidase A and neuraminidase H, three additional primer pairs were designed using the sequence data given by Yasawong et al. (2010) and the NCBI primer design program. The oligonucleotide primers had the sequences TTCTCACCTTCACCTTCGCC and TACCCGAATGAT GCGATGGG (collagen binding protein), ACCGTGAAGG TTAACGCCAT and CTTGGTGTCTGCCGTGATCT (neuraminidase A), GCCAACAGCAACCTTGTCAG and CAAACGCCGCCAAGAAGAAA (neuraminidase H) with the PCR program: 1× (94 °C, 180 s), 30× (94 °C, 45 s, 54 °C, 30 s, 72 °C, 60 s), 1× (72 °C, 420 s).

All six *A. haemolyticum* strains of the present study produced a narrow zone of complete hemolysis on sheep blood agar and, compared to sheep blood, an enhanced hemolysis on rabbit blood agar, a synergistic CAMP-like reaction with *Streptococcus agalactiae* and *Rhodococcus equi* as indicator strains, a reverse CAMP reaction in the area of staphylococcal β-hemolysin and had the typical biochemical properties of this species (Table 2). The hemolytic properties on rabbit blood agar, the CAMP-like activities and the reverse CAMP reaction are known to be typical properties of *A. haemolyticum* (Lämmler and Hartwigk 1995; Hassan et al. 2009; Ülbegi-Mohyla et al. 2009; Hijazin et al. 2010; Brown et al. 2013). All six *A. haemolyticum* strains showed a cross-reaction with streptococcal serogroup B-specific antiserum which is also known as typical property of *A. haemolyticum* (García-de-la-Fuente et al. 2012; Brown et al. 2013). As shown by numerous authors MALDI-TOF MS appears to be a powerful tool for species classification of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al. 2009; Murray 2010). Comparable to previous MALDI-TOF MS analysis of bacteria of genera *Arcanobacterium* and *Trueperella* (Hijazin et al. 2012a) and to a previously investigated *A. haemolyticum* strain isolated from a donkey (Sammra et al. 2014), MALDI-TOF MS allowed the identification of all six *A. haemolyticum* of the present study to the species level matching to *A. haemolyticum* in the data base with log score values ≥2 (data not shown). A dendrogram of the MALDI-TOF MS results of the six *A. haemolyticum* strains of the present study, *A. haemolyticum* DSM 20595 and various other species of genus *Arcanobacterium* is shown in Fig. 1.

Table 1 Origin of the six *A. haemolyticum* strains investigated in the present study

Patient	Clinical symptoms and underlying disease	*A. haemolyticum* strain designation	Date of isolation (day.month.year)	Origin	Other isolated microorganism(s)
1	Appendicitis	7-2596628	22.03.12	Pus from periappendicular abscess	*Fusobacterium necrophorum*
2	Chronic osteomyelitis of one toe; Diabetes 2	7-4438845	04.07.11	Biopsy from osteomyelitis	*Streptococcus agalactiae; Enterococcus. faecalis; Staphylococcus aureus*; Coagulase negative staphylococci
3	Abscess dorsum pedis, phlegmone of leg; Diabetes 1	7-4823400	05.08.11	Biopsy from abscess	Streptococci of serogroup G; *S. aureus*
4	Chronic plantar abscess; Cirrhosis of the liver	7-4991567	17.12.11	Biopsy from abscess	*E. faecalis*; *Enterobacter cloacae*
5	Chronic abscess in calcaneus; Alcoholism	E2-1797395	16.04.12	Biopsy from the heel	*Prevotella bergensis*
6	Chronic osteomyelitis of one toe; Alcoholism	E2-1942131	30.04.12	Biopsy from osteomyelitis	Streptococci of serogroup G; *S. aureus*

Table 2 Phenotypical and genotypical properties of the six *A. haemolyticum* strains investigated in the present study and *A. haemolyticum* DSM 20595

Phenotypic properties	*A. haemolyticum* (*n*=6)	*A.haemolyticum* DSM 20595**
Hemolysis on sheep blood agar	+(6)	+
Hemolysis on rabbit blood agar	++(5); +(+)(1)	++
CAMP-like reaction with:*		
Streptococcus agalactiae	+(4); (+)(2)	+
Rhodococcus equi	+(5); (+)(1)	+
Reverse CAMP reaction	+(4); (+)(2)	+
Nitrate reduction	−(5)[a]; +(1)[a]	−[a]
Pyrazinamidase	+(6)[a]	+[a]
Pyrrolidonyl arylamidase	−(5)[a]; (+)(1)[a]	−[a]
Alkaline phosphatase	+(6)[a]	+[a]
β-Glucuronidase (β-GUR)	−(4)[a,b,c]; +(2)[a,b,c]	−[a,b,c]
α-Galactosidase (α-GAL)	−(6)[b]	−[b]
β-Galactosidase (β-GAL)	+(6)[a,c]	+[a,c]
α-Glucosidase (α-GLU)	+(6)[a,b,c]	+[a,b,c]
β-Glucosidase (β-GLU)	−(6)[b]	−[b]
N-Acetyl-β-glucosaminidase (β-NAG)	+(6)[a,c]	+[a,c]
Esculin (β-glucosidase)	−(6)[a]	−[a]
Urease	−(6)[a]	−[a]
Gelatine	−(6)[a]	−[a]
Fermentation of:		
Glucose	+(6)[a]	+[a]
Ribose	+(6)[a]	+[a]
Xylose	−(6)[a]	−[a]
Mannitol	−(6)[a]	−[a]
Maltose	+(3)[a];(+)(3)[a]	+[a]
Lactose	+(6)[a]	+[a]
Saccharose	−(6)[a]	−[a]
Glycogen	−(6)[a]	−[a]
α-Mannosidase	+(6)[b]	+[b]
Catalase	− (6)	−
Serolysis on Loeffler agar	−(6)	−
Caseinase	−(6)	−
DNase	+(6)	+
Starch hydrolysis	+(3); −(3)	−
Cross-reaction with streptococcal serogroup B-specific antiserum	+(6)	+
Molecular identification		
16S rDNA	+	+
gap	+	+
ISR-23S rDNA	+	+
Genes encoding the potential virulence factors		
Arcanolysin	+(6)	+
Phospholipase D	+(6)	+
Hemolysin A	+(6)	+
CAMP factor family protein	+(6)	+
Collagen binding protein	+(2); −(4)	+
Neuraminidase A	+(4); −(2)	+
Neuraminidase H	+(4); −(2)	+

The reactions are shown as follows:

*, synergistic CAMP-like reaction with indicator strains

**, results mostly obtained from Hassan et al. (2009) and Hijazin et al. (2010)

++, enhanced positive reaction

+(+), moderately enhanced positive reaction

+, positive reaction

(+), weak positive reaction

−, negative reaction

[a] Api-Coryne test system (Biomerieux, Nürtingen, Germany)

[b] Tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark)

[c] 4-Methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany)

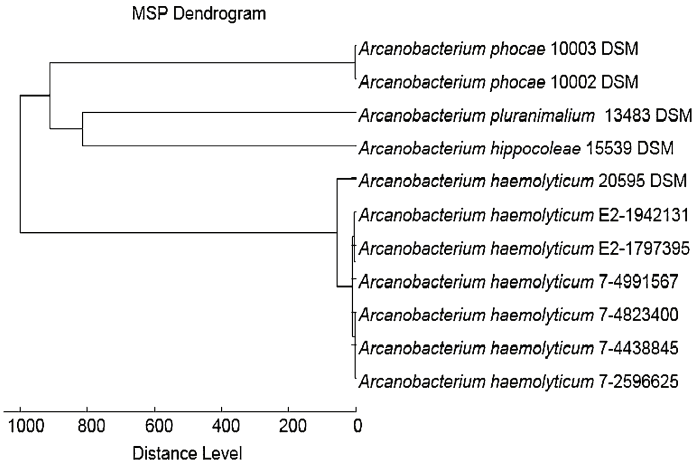

Fig. 1 MALDI-TOF MS spectra of the six *A. haemolyticum* strains of the present study, reference strain *A. haemolyticum* DSM 20595 and various other species of genus *Arcanobacterium*

Sequencing 16S rDNA and gene *gap* of all six *A. haemolyticum* strains of the present study yielded a sequence identity of ≥99.2 % to 16S rDNA and gene *gap* sequence of *A. haemolyticum* DSM 20595, respectively. A

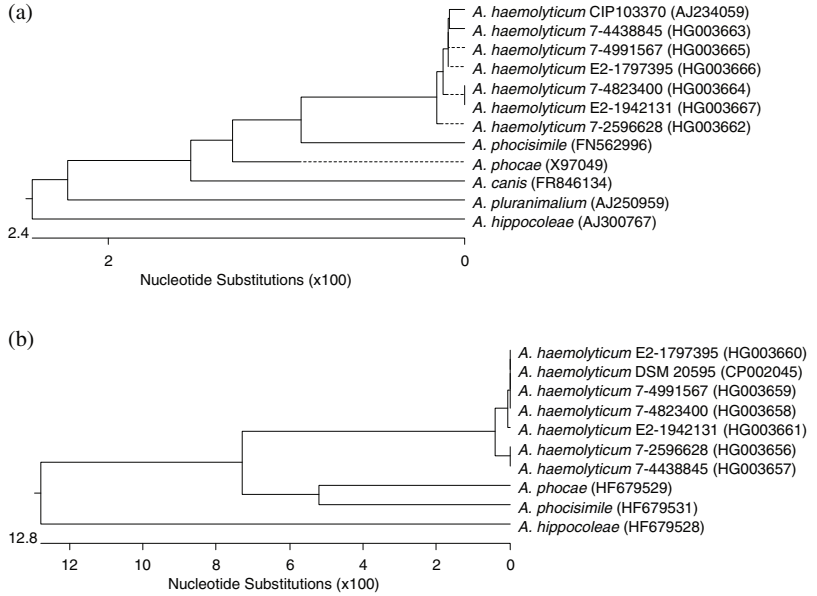

Fig. 2 Dendogram analysis of the 16S rDNA (**a**) and gene *gap* (**b**) sequences of *A. haemolyticum* 7-2596628 (HG003662, HG003656), *A. haemolyticum* 7-4438845 (HG003663, HG003657), *A. haemolyticum* 7-4823400 (HG003664, HG003658), *A. haemolyticum* 7-4991567 (HG003665, HG003659), *A. haemolyticum* E2-1797395 (HG003666, HG003660) and *A. haemolyticum* E2-1942131 (HG003667, HG003661) of the present study and various species of genus *Arcanobacterium* obtained from NCBI GenBank

dendrogram analysis of both sequences is shown in Fig. 2. The species identity of all six *A. haemolyticum* could also be confirmed by amplification of an *A. haemolyticum* specific region of ISR and 23S rDNA (Table 2). The usefulness of this species-specific PCR had already been shown for *A. haemolyticum* of horse and donkey origin (Hijazin et al. 2010; Sammra et al. 2014).

Further studies investigating the seven potential virulence genes encoding arcanolysin, phospholipase D, hemolysin A, CAMP factor family protein, a collagen binding protein and two neuraminidases of *A. haemolyticum* revealed the presence of all seven virulence genes in two strains, six virulence genes in two strains and the presence of four virulence genes in two strains, respectively. The positive and negative results which could be observed with the oligonucleotide primers amplifying the genes encoding collagen binding protein, neuraminidase A and neuraminidase H could be observed with the oligonucleotide primers described previously (Sammra et al. 2014) and with the newly described oligonucleotide primers designed in the present study indicating that the negative reactions were not caused by a point mutation of the primer binding sites but a lack of the genes, respectively (Table 2). In 2011, Jost et al. characterized arcanolysin as a cholesterol-dependent cytolysin with close relation to pyolysin of *T. pyogenes*. Recombinant arcanolysin displayed hemolytic activities on erythrocytes, more pronounced on rabbit and human erythrocytes, and cytolytic activities on cell lines of various origins. Comparable to the present study, Jost et al. (2011) could detect arcanolysin encoding gene *aln* in all 52 *A. haemolyticum* strains investigated. In addition, Lucas et al. (2010) demonstrated that phospholipase D encoding gene *pld* could be found in all *A. haemolyticum* strains and that phospholipase D enhances bacterial adhesion and promotes host cell necrosis following invasion. A collagen binding protein and two different neuraminidases were already described as potential virulence factors of *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) (Jost and Billington 2005) and more recently also as potential virulence factors of *A. haemolyticum* (Lucas 2009). Little is known about the role the other potential virulence factors play in infection processes. The present study gives initial information about the presence and absence of seven potential virulence factor encoding genes of *A. haemolyticum* isolated from human patients. Further studies will give information about the presence or absence of these virulence genes and clinical symptoms. However, the phenotypic and genotypic properties described in the present study could be used to identify and further characterize *A. haemolyticum* isolated from human origin. This might help to give the true prevalence of this bacterial species which seems to be of increasing importance. As seen in Table 1, all six *A. haemolyticum* investigated in the present study were isolated from the six patients as polymicrobial community together with various other potentially pathogenic bacterial species indicating that the importance of the *A. haemolyticum* for the clinical symptoms and the underlying diseases of the six patients remains unclear.

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Short Communication

Identification of *Arcanobacterium pluranimalium* by matrix-assisted laser desorption ionization-time of flight mass spectrometry and, as novel target, by sequencing pluranimaliumlysin encoding gene *pla*

A. Balbutskaya [a], O. Sammra [a], S. Nagib [a], M. Hijazin [a], J. Alber [a], C. Lämmler [a,*], G. Foster [b], M. Erhard [c], P.N. Wragg [d], A. Abdulmawjood [e], E. Prenger-Berninghoff [f]

[a] Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Schubertstr 81, 35392 Gießen, Germany
[b] SAC Consulting Veterinary Services, Drummondhill, Stratherrick Road, Inverness IV2 4JZ, UK
[c] RIPAC-LABOR GmbH, Am Mühlenberg 11, 14476 Potsdam-Golm, Germany
[d] Animal Health and Veterinary Laboratories Agency, Penrith Regional Laboratory, Merrythought, Calthwaite, Penrith CA11 9RR, UK
[e] Institut für Lebensmittelqualität und-sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany
[f] Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurterstr. 85-91, 35392 Gießen, Germany

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ABSTRACT

In the present study 13 *Arcanobacterium pluranimalium* strains isolated from various animal origin could successfully be identified phenotypically by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and genotypically by sequencing 16S rDNA and the pluranimaliumlysin encoding gene *pla*. The detection of mass spectra by MALDI-TOF MS and the novel genotypic approach using gene *pla* might help to identify *A. pluranimalium* in future and might elucidate the role this species plays in infections of animals.

1. Introduction

According to a proposal of Yassin et al. (2011) genus *Arcanobacterium* consists of four species namely *Arcanobacterium haemolyticum*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae*. More recently *Arcanobacterium canis* and *Arcanobacterium phocisimile* were described as novel species of genus *Arcanobacterium* (Hijazin et al., 2012a, 2013). The closely related species *Arcanobacterium pyogenes*, *Arcanobacterium abortisuis*, *Arcanobacterium bernardiae*, *Arcanobacterium bialowiezense* and *Arcanobacterium bonasi* were reclassified to the newly described genus *Trueperella* as *Trueperella pyogenes*, *Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis* and *Trueperella bonasi* (Yassin et al., 2011). However, both genera belong to the family *Actinomycetaceae* (http://www.ncbi.nlm.nih.gov/taxonomy).

A. pluranimalium was initially described based on two isolates which were recovered from a harbour porpoise and a fallow deer, respectively (Lawson et al., 2001). In the

* Corresponding author. Tel.: +49 6419938406; fax: +49 6419938409.
E-mail address: christoph.laemmler@vetmed.uni-giessen.de (C. Lämmler).

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following years this bacterial species was isolated in a mixed culture from a dog with pyoderma (Ülbegi-Mohyla et al., 2010a) and from abortion material, semen, abscesses, viscera, navel ill and peritonitis of sheep and from a milk sample of a cow with mastitis (Foster and Hunt, 2011). The identification of the hitherto described *A. pluranimalium* was performed with phenotypic methods (Foster and Hunt, 2011) and by sequencing 16S rDNA, 16S-23S rDNA intergenic spacer region (ISR) and 23S rDNA (Lawson et al., 2001; Ülbegi-Mohyla et al., 2010a).

The present study was designed to investigate the usefulness of MALDI-TOF MS and the pluranimaliumlysin encoding target gene *pla* for identification of novel and previously described *A. pluranimalium* strains of various origins.

2. Materials and methods

The strains used in the present study included reference strain *A. pluranimalium* DSM 13483, other strains of genus *Arcanobacterium* (Sammra et al., 2014), *A. pluranimalium* 1128 isolated from a dog with pyoderma (Ülbegi-Mohyla et al., 2010a) and 12 hitherto not characterized *A. pluranimalium* strains isolated in Scotland, England and Germany. Further data about the origin of the 13 *A. pluranimalium* strains are summarized in Table 1.

The strains were investigated phenotypically (Ülbegi-Mohyla et al., 2010a; Foster and Hunt, 2011), by 16S rDNA sequencing (Sammra et al., 2014) and by MALDI-TOF MS analysis as described previously (Hijazin et al., 2012b).

In addition the previously sequenced pluranimaliumlysin encoding gene *pla* of *A. pluranimalium* DSM 13483 (FR745890) was used for the design of *pla* specific oligonucleotide primer. The oligonucleotide primer had the sequence *pla*-F 5′-GTT GAT CTA CCA GGA TTG ACG C-3′ and *pla*-R 5′-TTG TCG GGG TGT CCA TTG CC-3′ and were used with the following PCR program: one step of 3 min at 94 °C; 30 cycles, with 1 cycle consisting of 45 s at 94 °C, 30 s at 57 °C, and 60 s at 72 °C; and one step of 7 min at 72 °C.

Sequencing was performed by Seqlab-Sequence Laboratories, Göttingen, Germany, alignment studies and dendrogram analysis using DNASTAR Lasergene Version 8.0.2 (DNASTAR Inc., Madison, USA) by Clustal W method.

Table 1
Origin of the 13 *A. pluranimalium* strains investigated in the present study.

A. pluranimalium strain designation	Isolated from
S111911-12-1	Ovine placenta, S
S214809-11-1	Ovine foetal stomach contents, S
S214961-11-1	Ovine vaginal swab, S
S215069-11-1	Ovine foetal stomach contents, S
S420838-12-1	Ovine foetal stomach contents, S
S609650-12-1	Ovine foetal stomach contents, S
12-S0201-04-12	Ovine milk, E
15-S38-11-10	Ovine brisket abscess, E
C632524-10-1	Bovine foetal stomach contents, S
24-C359-11-10	Bovine foetal stomach contents, E
D12-0439-1-1-1	Bovine mastitis, G
D13-0340-5-1-1	Bovine cervix, G
1128*	Dog with pyoderma, G

S = Scotland, E = England, G = Germany.
* Ülbegi-Mohyla et al. (2010a).

3. Results and discussion

All 12 *A. pluranimalium* strains newly investigated in the present study showed the typical phenotypical properties of this species and could be classified as *A. pluranimalium* by 16S rDNA sequencing. A typical dendrogram of the 16S rDNA sequencing results is shown in Fig. 1. The phenotypical properties included a moderate hemolysis on sheep blood agar, a slightly enhanced hemolysis on rabbit blood agar, positive CAMP-like reactions with *Staphylococcus aureus* β-hemolysin, *Rhodococcus equi*

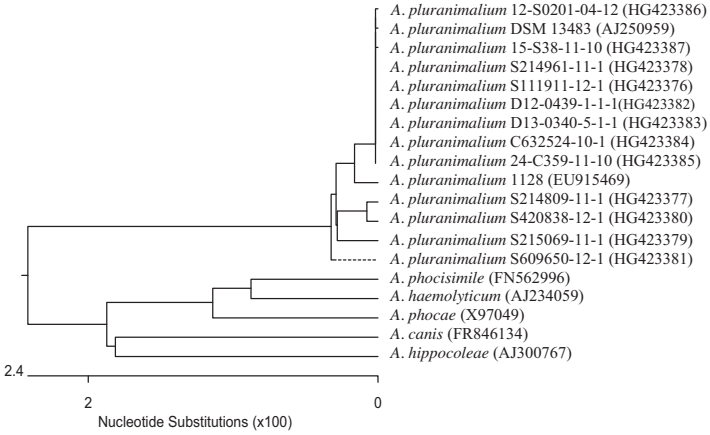

Fig. 1. Dendrogram analysis of 16S rDNA sequences of the 13 *A. pluranimalium* strains of the present study, reference strain *A. pluranimalium* DSM 13483 and of various other species of genus *Arcanobacterium* obtained from NCBI GenBank.

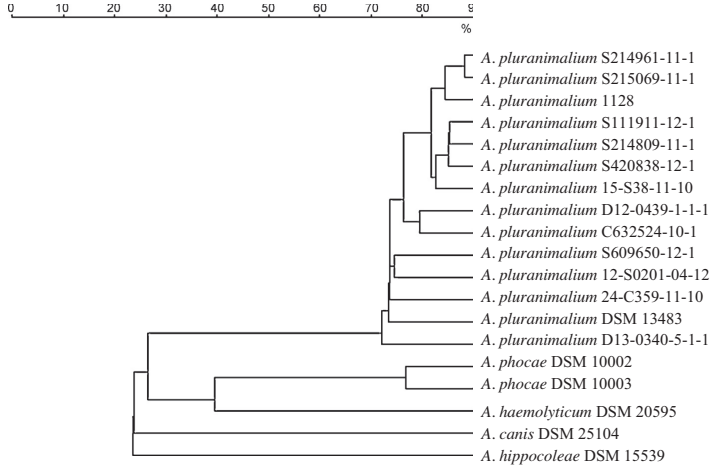

Fig. 2. MALDI-TOF MS spectra from 13 *A. pluranimalium* strains of the present study, reference strain *A. pluranimalium* DSM 13483 and various other species of the genus *Arcanobacterium*.

and *Arcanobacterium haemolyticum* as indicator strains, generally positive reactions for caseinase ($n = 10$), catalase ($n = 8$), starch hydrolysis ($n = 12$), β-glucuronidase ($n = 12$) and negative reactions for α-glucosidase ($n = 12$) and *N*-acetyl-β-glucosaminidase ($n = 12$). It was of interest that all eight *A. pluranimalium* strains of ovine origin and the previously described *A. pluranimalium* of canine origin (Ülbegi-Mohyla et al., 2010a) were catalase positive whereas the four strains of bovine origin were catalase negative (data not shown).

Comparable to previously conducted MALDI-TOF MS analysis of a *Trueperella* (*Arcanobacterium*) *bernardiae* strain of animal origin (Hijazin et al., 2012b), MALDI-TOF MS allowed the identification of all 12 newly described *A. pluranimalium* strains in the present study and *A. pluranimalium* 1128 of canine origin to the species level (Fig. 2.), indicating, that MALDI-TOF MS could also be used to reliably identify *A. pluranimalium* strains isolated from various origins. As shown by numerous authors MALDI-TOF MS appears to be a powerful tool

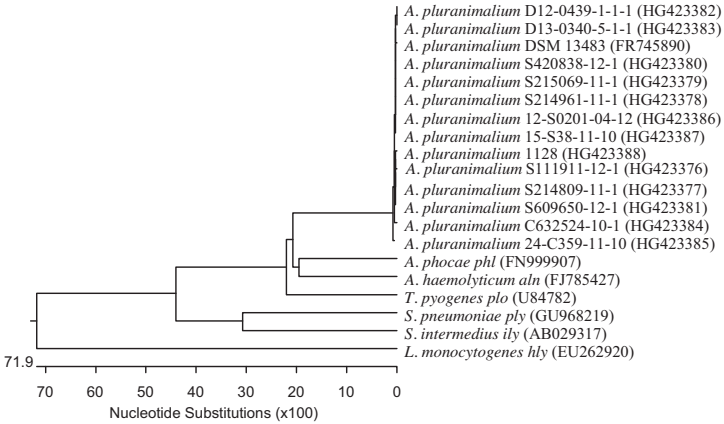

Fig. 3. Dendrogram analysis of *pla* gene sequences of the 13 *A. pluranimalium* strains of the present study, *pla* of *A. pluranimalium* DSM 13483 and the genes *plo* of *T. pyogenes*, *aln* of *A. haemolyticum*, *phl* of *A. phocae*, *ply* of *Streptococcus pneumoniae*, *ily* of *Streptococcus intermedius* and *hly* of *Listeria monocytogenes* obtained from NCBI GenBank.

for species classification of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011).

Using the *pla* specific oligonucleotide primer which were based on the previously published partial gene sequence (FR745890) *pla* of all 13 *A. pluranimalium* strains investigated in the present study could be amplified and sequenced yielding 98,9% identity of the *pla* sequences among each other. Dendrogram analysis of *pla* of *A. pluranimalium*, *plo* of *T. pyogenes*, *phl* of *A. phocae*, *aln* of *A. haemolyticum* and of other pore forming toxin encoding genes obtained from NCBI GenBank revealed a close relation of *pla*, *plo*, *phl* and *aln* (Fig. 3). Comparable to gene *plo* of *T. pyogenes*, which appeared to be a constant characteristic of all investigated *T. pyogenes* (Billington et al., 1997; Ertaş et al., 2005; Ülbegi-Mohyla et al., 2010b; Hijazin et al., 2011), *pla* of *A. pluranimalium* seems to be also constantly present in all strains of this species and could be used for molecular identification of *A. pluranimalium*. More recently Moser et al. (2013) also described *pla* as a novel target for molecular identification of this species. Further studies will give information about the consistent presence and the pathogenic importance of this hitherto unknown virulence factor encoding gene of *A. pluranimalium*.

A. pluranimalium had already been characterized by amplification and sequencing of species specific regions of ISR and 23S rDNA (Ülbegi-Mohyla et al., 2010a) and by sequencing of the target genes *rpoB*, *sodA*, *cbpA* and *gap* (Hijazin et al., 2011; Sammra et al., 2014).

The MALDI-TOF MS analysis of the present study and the target gene *pla* might improve the identification of *A. pluranimalium* and might help to elucidate the role this species plays in infections of sheep, bovines, dogs, other animals and possibly in humans.

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Research Article

Phenotypical and Genotypical Properties of an *Arcanobacterium pluranimalium* Strain Isolated from a Juvenile Giraffe (*Giraffa camelopardalis reticulata*)

Karin Risse,[1] Karen Schlez,[1] Tobias Eisenberg,[1] Christina Geiger,[2] Anna Balbutskaya,[3] Osama Sammra,[3] Christoph Lämmler,[3] and Amir Abdulmawjood[4]

[1] *Landesbetrieb Hessisches Landeslabor, Schubertstraße 60, 35392 Gießen, Germany*
[2] *Frankfurt Zoo, Bernhard-Grzimek-Allee 1, 60316 Frankfurt, Germany*
[3] *Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Schubertstraße 81, 35392 Gießen, Germany*
[4] *Institut für Lebensmittelqualität und-sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany*

Correspondence should be addressed to Christoph Lämmler; christoph.laemmler@vetmed.uni-giessen.de

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The present study was designed to characterize phenotypically and genotypically an *Arcanobacterium pluranimalium* strain (*A. pluranimalium* 4868) following necropsy from a juvenile giraffe. The species identity could be confirmed by phenotypical investigations and by MALDI-TOF MS analysis, by sequencing the 16S rDNA, pluranimaliumlysin encoding gene *pla*, and glyceraldehyde-3-phosphate dehydrogenase encoding gene *gap* with sequence similarities to *A. pluranimalium* reference strain DSM 13483^T of 99.2%, 89.9%, and 99.1%, respectively. To our knowledge, the present study is the first phenotypic and genotypic characterization of an *A. pluranimalium* strain isolated from a giraffe.

1. Introduction

Genus *Arcanobacterium* was described by Collins et al. 1982 [1] as a group of facultative anaerobic, asporogenous, and Gram-stain positive rods. According to Yassin et al. (2011) [2], this genus consists of four species, namely, *Arcanobacterium haemolyticum*, *Arcanobacterium hippocoleae*, *Arcanobacterium phocae,* and *Arcanobacterium pluranimalium*. More recently, *Arcanobacterium canis* and *Arcanobacterium phocisimile*, two species which were most closely related to *A. haemolyticum*, were described as novel species of this genus [3, 4].

The original species characterization of *A. pluranimalium* was performed with two strains isolated from a dead harbour porpoise and a dead fallow deer [5]. In the following years *A. pluranimalium* could also be isolated from a dog with pyoderma [6], from ovine specimens on 33 occasions, and from a milk sample of a single cow with mastitis [7]. More recently several *A. pluranimalium* strains recovered from various specimens were identified phenotypically and by using various molecular targets [8].

2. Material and Methods

The present study was focused on the characterization of an *A. pluranimalium* strain following necropsy from a juvenile giraffe by various phenotypic properties, by MALDI-TOF MS analysis, and genotypically by sequencing 16S rDNA and the *A. pluranimalium*-specific target genes *pla* and *gap*.

The 80.5 kg female giraffe (*Giraffa camelopardalis reticulata*) of the present study was born in 2013. The giraffe was not accepted by its mother or wet nurse and did not

accept hand rearing attempts and, because of general weakness, was euthanized three days after birth. The subsequent postmortem analysis revealed an acute hyperemia of lung and liver and a focal emphysema of the lung. The acute pneumonia was caused by a bacterial infection associated with aspirated foreign bodies.

Bacteriological investigations yielded the isolation of *A. pluranimalium* and *Escherichia coli*, partly together with coagulase negative staphylococci, α-haemolytic streptococci, and *Pseudomonas fluorescens* from liver, spleen, kidney, and lung. A moderate to high growth of *E. coli* was generally noted (++, +++); *A. pluranimalium* grew only in low numbers (+). The *A. pluranimalium* strain 4868, originally obtained from the spleen, was used for further studies. The bacterial strain was investigated phenotypically and by MALDI-TOF analysis [6, 9] and genotypically by amplification and sequencing of 16S rDNA using universal oligonucleotide primer 16 UNI-L (5′-AGA-GTT-TGA-TCA-TGG-CTC-AG-3) and 16 UNI-R (5′-GTG-TGA-CGG-GCG-GTG-TGT-AC-3) for amplification, under the following PCR conditions: (×1 (95°C, 600 sec), ×30 (95°C, 30 sec, 58°C, 60 sec, 72°C, 60 sec), and using oligonucleotide primer 533-F (5′-GTG-CCA-GCM-GCC-GCG-GTA-A′-3) and 907R (5′-CCG-TCA-ATT-CMT-TTG-AGT-TT-3′) for sequencing. The strain was also characterized by amplification of the target gene *pla* with the oligonucleotide primer *pla*-F: 5′-GTT GAT CTA CCA GGA TTG ACG C-3′ and *pla*-R: 5′-TTG TCG GGG TGT CCA TTG CC-3′ and gene *gap* with the oligonucleotide primer *gap*-F 5′-TTG ACC GAC AAC AAG ACC CT-3′ and *gap*-R 5′-CCA TTC GTT GTC GTA CCA AG-3′as described [8, 10]. Alignment studies were performed using DNASTAR Lasergene Version 8.0.2 (DNASTAR Inc., Madison, WI, USA), Clustal W method. For MALDI-TOF MS the isolates were prepared using the direct smear method as well as an extraction protocol provided by the manufacturer. Briefly, freshly grown bacteria were harvested and diluted in ethanol, centrifuged (2000 ×g), air-dried, and resuspended in aqueous volumes of 70% formic acid and acetonitril followed by a vortex step. Five microliters was directly transferred to the steel target. Analysis was performed on a MALDI-TOF MS Biotyper Version V3.3.1.0. The database used (DB 4613, Bruker Daltonics) comprised 45 spectra from *A. pluranimalium* DSM 13483^{T}.

3. Results and Discussions

A. pluranimalium 4868 investigated in the present study was identified by determination of hemolysis and CAMP-like hemolytic reactions, by using a commercial identification system as well as various other phenotypical tests. The CAMP-like hemolytic reactions with *Staphylococcus aureus* β-hemolysin, *Rhodococcus equi,* and *Arcanobacterium haemolyticum* as indicator strains are known as typicalcharacteristics of this species [6, 8, 11]. Comparable to previously investigated *A. pluranimalium* [6, 8] the phenotypical tests also revealed the typical biochemical properties of this species (Table 1). It was of interest that *A. pluranimalium* 4868 of the present study was catalase negative. This was observed previously for *A. pluranimalium* of bovine origin [8].

As shown by numerous authors MALDI-TOF MS is a powerful tool for species identification of a broad spectrum of bacteria including Gram-positive and Gram-negative bacteria [12–14]. Comparable to the previously conducted MALDI-TOF MS analysis of bacteria of genera *Arcanobacterium* and *Trueperella* (formerly belonging to genus *Arcanobacterium* [9, 15]), MALDI-TOF MS allowed the identification of *A. pluranimalium* 4868 of the present study to the species level matching to *A. pluranimalium* reference strain DSM 13483^{T} with a log score value of 2.28.

Sequencing 16S rDNA, the potentially cytolytic toxin pluranimaliumlysin encoding target gene *pla* and the glyceraldehyde-3-phosphate dehydrogenase encoding target gene *gap* revealed a sequence similarity of 99.2%, 89.9%, and 99.1% to the respective sequences of *A. pluranimalium* DSM 13483^{T}. All three sequences of *A. pluranimalium* 4868 were deposited in GenBank (HG794511, HG423389, and HG423390). A typical dendrogram of the sequencing results of the genes *pla* and *gap* is shown in Figures 1 and 2. Comparable to gene *plo* of *T. pyogenes*, which appeared to be a constant characteristic of all investigated *T. pyogenes* [16–19], *pla* of *A. pluranimalium* seems to be also constantly present in all strains of this species and could be used, as described previously [8], and in the present study for molecular identification of *A. pluranimalium*. More recently, Moser et al. 2013 [20] also described *pla* as novel target for molecular identification of this species.

Sequencing of gene *gap* had already been described for molecular identification of staphylococcal species [21] and more recently for identification of an *A. haemolyticum* strain isolated from a donkey [10]. In the present study gene *gap* could also be used as novel target for identification of *A. pluranimalium*. Further studies will give information about the constant presence and sequence similarities of both target genes *pla* and *gap*, respectively.

4. Conclusion

The clinical importance of *A. pluranimalium* of the present study, which was isolated from various organs of the giraffe together with in high number appearing *E. coli*, remains unclear. Since, beside aspiration pneumonia, no other pathological findings could be detected, this might represent the route of infection. However, the isolation of this bacterial species from giraffe and the hitherto described origin harbor porpoise, fallow deer, dog, sheep, and cow emphasizes the species name *A. pluranimalium*.

Table 1: Biochemical properties of *A. pluranimalium* 4868 investigated in the present study and *A. pluranimalium* DSM 13483^{T}.

Biochemical properties	*A. pluranimalium* 4868	*A. pluranimalium* DSM 13483^{T**}
Hemolysis on sheep blood agar	+	+
CAMP-like reaction with:*		
Staphylococcus aureus β-hemolysin	+	+
Streptococcus agalactiae	−	−
Rhodococcus equi	+	+
Arcanobacterium haemolyticum	+	+
Reverse CAMP reaction	−	−
Nitrate reduction	$-^{1}$	$-^{1}$
Pyrazinamidase	$+^{1}$	$+^{1}$
Pyrrolidonyl arylamidase	$+^{1}$	$+^{1,2}$
Alkaline phosphatase	$-^{1}$	$-^{1,2}$
β-Glucuronidase (β-GUR)	$+^{1,2,3}$	$+^{1,2,3}$
β-Galactosidase (β-GAL)	$-^{1}$, $(+)^{3}$	$-^{1}$, $(+)^{3}$
α-Glucosidase (α-GLU)	$-^{1,2,3}$	$-^{1,2,3}$
β-Glucosidase (β-GLU)	$+^{2}$	$+^{2}$
N-Acetyl-β-glucosaminidase (β-NAG)	$-^{1,3}$	$-^{1,3}$
Esculin (β-glucosidase)	$(+)^{1}$	$+^{1}$
Urease	$-^{1}$	$-^{1}$
Gelatine	$+^{1}$	$+^{1}$
Fermentation of:		
Glucose	$+^{1}$	$+^{1}$
Ribose	$+^{1}$	$+^{1}$
Xylose	$(+)^{1}$	$-^{1}$
Mannitol	$-^{1}$	$-^{1}$
Maltose	$-^{1}$	$(+)^{1}$
Lactose	$-^{1}$	$-^{1}$
Saccharose	$-^{1}$	$-^{1}$
Glycogen	$-^{1}$	$-^{1}$
α-Mannosidase	$-^{2}$	$+^{2}$
Catalase	−	+

The reactions are shown as follows: *synergistic CAMP-like reaction with indicator strains; **results mostly obtained from Ülbegi-Mohyla et al., 2010 [6]; +: positive reaction; (+): weak positive reaction; −: negative reaction. [1]Api-Coryne test system (Biomerieux, Nürtingen, Germany); [2]tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); [3]4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

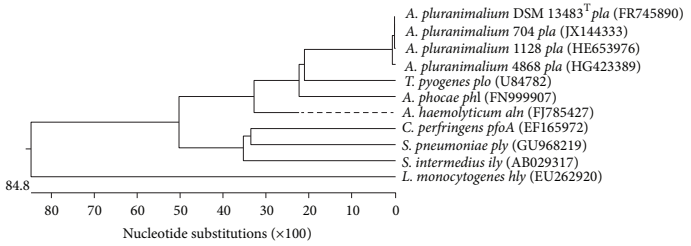

Figure 1: Dendrogram of sequences of gene *pla* of *A. pluranimalium* 4868 of the present study, three additional *A. pluranimalium*, and various other cytolytic toxin encoding genes obtained from GenBank.

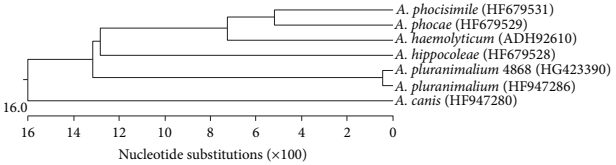

Figure 2: Dendrogram of gene *gap* of *A. pluranimalium* 4868, reference strain *A. pluranimalium* DSM 13483^T, and various other species of genus *Arcanobacterium* obtained from GenBank.

Conflict of Interests

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in this paper.

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Research - Full Paper

Phenotypic and Genotypic Analysis of an *Arcanobacterium pluranimalium* Isolated from a Muskox (*Ovibos moschatus*)

Siti Gusti Ningrum[1*], Wyanda Arnafia[1], Osama Sammra[2], Mazen Alssahen[2], Jörn-Peter Wickhorst[2], Abdulwahed Ahmed Hassan[3], Christoph Lämmler[2], Ellen Prenger-Berninghoff[4], Markus Timke[5], Amir Abdulmawjood[6]

Abstract
The present study was designed to characterize an *Arcanobacterium pluranimalium* strain isolated from a muskox (*Ovibos moschatus)* phenotypically, by MALDI-TOF MS analysis and genotypically using various molecular targets. The phenotypic properties, the MALDI-TOF MS analysis and sequencing the 16S rRNA gene, the β subunit of bacterial RNA polymerase encoding gene *rpoB*, the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap,* the elongation factor tu encoding gene *tuf* and the pluranimaliumlysin encoding gene *pla* allowed a successful identification of the isolated strain as *A. pluranimalium*. Gene *pla* could also be detected by a previously described loop-mediated isothermal amplification (LAMP) assay. This is first report on the isolation and characterization of *A. pluranimalium* originated from a Muskox.

Keywords
Arcanobacterium pluranimalium — muskox — MALDI-TOF MS — 16S rDNA — *rpoB*

[1] *Department of Animal Disease and Veterinary Public Health, Bogor Agricultural University, Bogor, 16680, Indonesia*
[2] *Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Giessen, Schubertstr. 81, 35392 Giessen, Germany*
[3] *Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq*
[4] *Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany*
[5] *Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany*
[6] *Institut für Lebensmittelqualität und-sicherheit, Stiftung Tierärztliche Hochschule Hannover, 30173 Hannover, Germany*
***Corresponding author**: sitiningrum10@gmail.com

Introduction

Arcanobacterium pluranimalium, belonging to family *Actinomycetaceae*, was initially characterized with two strains isolated from a dead harbor porpoise and a dead fallow dear (14). Further studies described the presence of this bacterial species from a dog with pyoderma (23), from a juvenile giraffe following necropsy (19), from ovine specimen on 33 occasions and from a milk sample of a cow with mastitis (7). Cases of bovine mastitis associated with *A. pluranimalium* were also described by Moser et al. (17), Balbutskaya et al. (3) and Wickhorst et al. (25). The present study gives a first report on the isolation and detailed characterization of an *A. pluranimalium* isolated from a muskox.

Material and Methods

The isolate investigated in the present study was obtained from a 19-year-old female muskox from Zoo of Cologne, Germany, which, after suffer from severe weakness, was euthanized in January 2015. Postmortem analysis revealed a diffuse catarrhal gastroenteritis, a *stomatitis profunda*, a diffuse fatty liver, and arthrosis of tarsal and carpal joints. Bacterial investigations yielded the isolation of *A. pluranimalium* and several other bacteria from the liver in low number and the isolation of aerobic bacilli, α-hemolytic streptococci and *Micrococcus* sp. from the intestine, also in low numbers.

The *A. pluranimalium* isolate was characterized by hemolysis on sheep blood agar, CAMP-like reaction, Reverse CAMP reaction, Api-Coryne test system (Biomerieux, Nürtingen, Germany), tablet containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark), 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany), catalase, Serolysis on Loeffler agar, caseinase and amylase determination according to previous studies (19, 23, 25). The reference strain used as positive control was obtained from Justus Liebig University bacterial collection.

MALDI-TOF MS analyses were performed according to the extraction protocol of previous study (3). A few colonies of freshly cultured bacteria were suspended into 75% ethanol. After centrifugation, the pellet was resuspended in 30 μl 70 % formic acid and with the same volume of pure acetonitrile. The suspension was centrifuged and 1 ml of the supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. The sample was overlaid with 1 ml matrix (10 mg α-cyano-4-hydroxy-cinnamic acid ml^{-1} in 50 % acetonitrile/2.5 % trifluoroacetic acid). Mass spectra were acquired using a microflex mass spectrometer (Bruker Daltonik) in the linear mode and a mass range of 2–20 kDa using the automated functionality of flexControl 3.0 software (Bruker Daltonik). At least 20 raw spectra were used

Table 1. Oligonucleotide primer sequences and PCR conditions

Oligonucleotide primers	Sequences	Program*	Size of PCR product (bp)	References
16S rDNA UNI-L 16S rDNA UNI-R	5'-AGAGTTTGATCATGGCTCAG-3' 5'-GTGTGACGGGCGGTGTGTAC-3	1	1352	9
*rpo*B-F *rpo*B-R	5'-CGWATGAACATYGGBCAGGT-3' 5'-TCCATYTCRCCRAARCGCTG-3'	2	406	24
gap-F *gap*-R	5'-TCGAAGTTGTTGCAGTTAACGA-3' 5'-CCATTCGTTGTCGTACCAAG-3'	3	784	20
tuf-F *tuf*-R	5'-GGACGGTAGTTGGAGAAGAATGG-3' 5'-CCAGGTTGATAACGCTCCAGAAGA-3'	4	796	25
pla-F *pla*-R	5'-GTTGATCTACCAGGATTGACGC-3' 5'-TTGTCGGGGTGTCCATTGCC-3'	5	283	3

*PCR program:

1: x1 (10 min at 95°C), x30 (30 s at 95°C, 60 s at 58°C, 60 s at 72°C), x1 (7 min at 72°C),

2: x1 (10 min at 95°C), x35 (30 s at 94°C, 30 s at 37°C, 120 s at 72°C), x1 (10 min at 72°C),

3: x1 (3 min at 94°C), x30 (30 s at 94°C, 40 s at 50°C, 60 s at 72°C), x1 (5 min at 72°C),

4: x1 (3 min at 94°C), x30 (45 s at 94°C, 40 s at 57°C, 60 s at 72°C), x1 (7 min at 72°C),

5: x1 (3 min at 94°C), x30 (45 s at 94°C, 30 s at 57°C, 60 s at 72°C), x1 (7 min at 72°C).

to generate a main spectrum. The main spectrum of the *A. pluranimalium* analysed in the present study was matched to the database included in the MALDI Biotyper 2.0 software package and to the newly acquired main spectra of reference strains representing six species of the genera *Arcanobacterium*. The software calculates a similarity score [log (score)] by calculation of a value considering the proportion of matching peaks between the unknown spectrum and the main spectrum of the database, the frequency of peaks in multiple measurements as well as the consistency of the peak intensities between these spectra. The logarithmized score values range from 0 (no similarity) to 3 (absolute identity). Log (score) values $\geq$2.3 are rated as highly probable species identification. Log (score) values 1.8-2.299 are considered as identification of microorganisms on the genus level and probable species level. Log (score) values 1.6-1.799 are considered as identification of microorganisms at least on the genus level. Log (score) values $<$1.6 indicate that a spectrum is not suitable for identification by the MALDI Biotyper.

16s rDNA, the β subunit of bacterial RNA polymerase encoding gene *rpoB*, the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap*, the elongation factor tu encoding gene *tuf* and the pluranimaliumlysin encoding gene *pla* were sequenced as described by Hassan et al. (9), Ülbegi-Mohyla et al. (24), Sammra et al. (20), Wickhorst et al. (25) and Balbutskaya et al. (3). A detailed description of the primer sequences and the temperature programs is listed in Table 1.

The presence of gene *pla* was determined with a previously described loop-mediated isothermal amplification (LAMP) assay (2). This was performed using a heat block and subsequent detection of the LAMP product in an agarose gel and by using a real-time fluorometer (Genie II®, Optigene, UK).

Results

The bacterial strain investigated in the present study could be identified phenotypically and genotypically as *A. pluranimalium*. The phenotypical test revealed the typical biochemical properties of this species (Table 2). According to MALDI-TOF MS analysis the investigated strain could be identified to the species level matching to type strain *A. pluranimalium* DSM 13483 with a log (score) value of 2.689 (Figure 1).

Sequencing 16S rDNA, the β subunit of bacterial RNA polymerase encoding gene *rpoB,* the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap*, the elongation factor to encoding gene *tuf* and pluranimaliumlysin encoding gene *pla* revealed a sequence identity of 99.9%, 99.8%, 99.9% 99.6%, and 99.6% to the respective sequences of type strain *A. pluranimalium* DSM 13483.

Typical dendrograms of the sequencing results of 16S rDNA and the genes *rpoB*, *gap*, *tuf* and *pla* are shown in Figure 2, 3 and 4. The species-specific gene *pla* of *A. pluranimalium* 230/15 of the present study could also successfully be detected by using a LAMP assay. The *pla* LAMP products could be detected as amplification signal using a real-time fluorometer (Figure 5).

Discussion and conclusion

A. pluranimalium strain 230/15 investigated in the present study was identified by determination of hemolysis, CAMP-like hemolytic reactions and by biochemical properties. The biochemical properties of the investigated strain showed the typical characteristics of this species.

Comparable to previously conducted MALDI-TOF MS analysis investigating bacteria of genus *Arcanobacterium, Trueperella* and *A. pluranimalium* of various origins (3, 10, 19, 25), the investigated strain of the present study could be clearly identified to the species level. MALDI-TOF MS has already been shown by numerous authors to constitute as rapid and reliable method for identification of various microorganisms (4, 18, 22).

In addition, several molecular targets have been exploited for molecular identification of this strain. The determination of sequences of various molecular targets is an important tool for identification of bacteria and for phylo-

Table 2. Biochemical properties of *A. pluranimalium* 230/15 investigated in the present study and type strain *A. pluranimalium* DSM 13483.

Biochemical properties	*A. pluranimalium* 230/15	*A. pluranimalium* DSM 13483**
Hemolysis on sheep blood agar	+	+
CAMP−like reaction with:*		
Staphylococcus aureus β−hemolysin	+	+
Streptococcus agalactiae	−	−
Rhodococcus equi	+	+
Reverse CAMP reaction	−	−
Nitrate reduction	−[1]	−[1]
Pyrazinamidase	+[1]	+[1]
Pyrrolidonyl arylamidase	(+)[1]	+[1]
Alkaline phosphatase	−[1]	−[1]
β−Glucuronidase	+[1,2,3]	+[1,2,3]
β−Galactosidase	−[1,2,3]	−[1,2,] (+)[3]
α−Glucosidase	−[1,2,3]	−[1,2,3]
β−Glucosidase	+[2]	+[2]
N−Acetyl−β−Glucosaminidase	−[1,3]	−[1,3]
Esculin	+[1]	+[1]
Urease	−[1]	−[1]
Gelatine	+[1]	+[1]
Fermentation of:		
D−Glucose	+[1]	+[1]
D−Ribose	+[1]	+[1]
D−Xylose	−[1]	−[1]
D−Mannitol	−[1]	−[1]
D−Maltose	(+)[1]	(+)[1]
D−Lactose	−[1]	−[1]
D−Saccharose	−[1]	−[1]
Glycogen	−[1]	−[1]
Catalase	+	+
Serolysis on Loeffler agar	−	−
Caseinase	+	+
Amylase	+	+

The reactions are shown as follows:

*synergistic CAMP−like reaction with indicator strains;

**results mostly obtained from Ülbegi−Mohyla et al. (23);

+: positive reaction; (+): weak positive reaction; −: negative reaction.

[1]Api−Coryne test system (Biomerieux, Nürtingen, Germany);

[2]tablet containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark);

[3]4−methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

genetic studies (8). Sequencing the 16S rDNA is a rDNA-based universal target among bacteria and large enough for bioinformatic purposes. Sequencing 16S rDNA of *A. pluranimalium* 230/15 of the present study showed a sequence similarity of 99.9% to 16S rDNA of type strain *A. pluranimalium* DSM 13483. The 16S rDNA contains various specific regions (5) and values of $\geq$ 99% similarity of 16S rDNA sequence were assigned as suitable cut-off for bacterial species identification and $\geq$ 97% for bacterial identification at genus level (6).

A molecular identification to the species level could also be conducted by amplification and sequencing the housekeeping genes *rpoB, gap* and *tuf* encoding the β subunit of bacterial RNA polymerase, the glyceraldehyde 3-phosphate dehydrogenase and the elongation factor tu. All three genes revealed a sequence identity of more than 99% indicating their usefulness as molecular targets. Khamis et al. (11) showed that gene *rpoB* could be used for phylogenetic analysis of species of genus *Corynebacterium*. In addition, gene *gap* has been widely used as target gene for molecular identification of various species of genus *Arcanobacterium* (20, 21) and genus *Staphylococcus* (8). The application of *tuf* gene analysis for molecular identification has already been evaluated for various bacterial species of genus *Enterococcus, Streptococcus,* coagulase-negative *Staphylococcus* and *Lactococcus* (15).

The investigated *A. pluranimalium* strain obtained from a muskox is also carrying the potential virulence factor pluranimaliumlysin encoding gene *pla*. Sequencing gene *pla* of *A. pluranimalium* 230/15 showed a high sequence similarity (99.6%) to *pla* gene of type strain *A. pluranimalium* DSM 13483. Gene *pla* has already been described as novel target for molecular identification of *A. pluranimalium* (17). Balbutskaya et al. (3), Risse et al. (19) and Wickhorst et

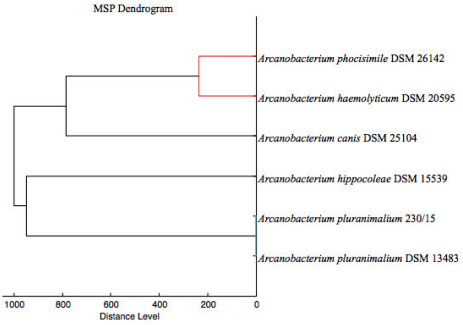

Figure 1. A score-oriented dendrogram of MALDI-TOF MS spectra profiles of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and some reference strains of genus *Arcanobacterium*

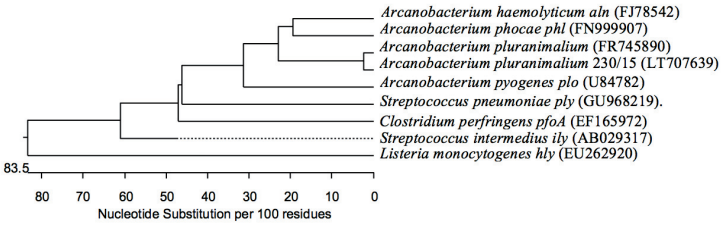

Figure 2. Dendrogram analysis of 16S rDNA of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and other species of genus *Arcanobacterium* obtained from NCBI GenBank. *Accession number

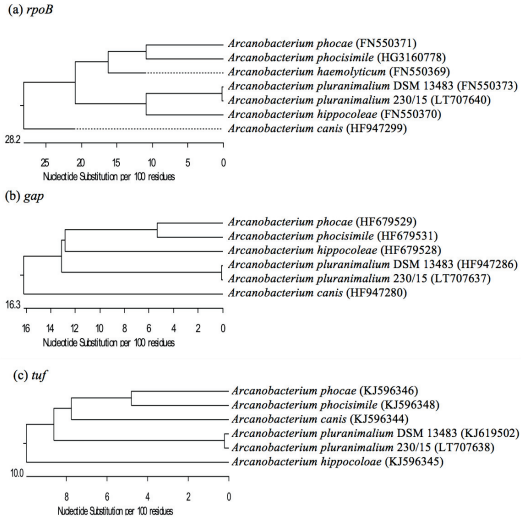

Figure 3. Dendrogram analysis of the genes *gap* (a) *rpoB* (b) and *tuf* (c) of *A. pluranimalium* 230/15, type strain *A. pluranimalium* DSM 13483 and other species of genus *Arcanobacterium* obtained from NCBI GenBank

al. (25) also showed that gene *pla* of *A. pluranimalium* seems to be constantly present in all strains of this species

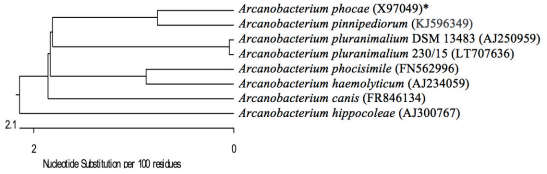

Figure 4. Dendrogram analysis of pluranimaliumlysin encoding gene *pla* of *A. pluranimalium* 230/15, *pla* of *A. pluranimalium* DSM 13483 and the genes *aln* of *A. haemolyticum, phl* of *A. phocae*, *plo* of *T. pyogenes*, *ily* of *Streptococcus intermedius*, *ply* of *Streptococcus pneumoniae*, *pfoA* of *Clostridium perfringens*, *hly* of *Listeria monocytogenes* obtained from NCBI GenBank

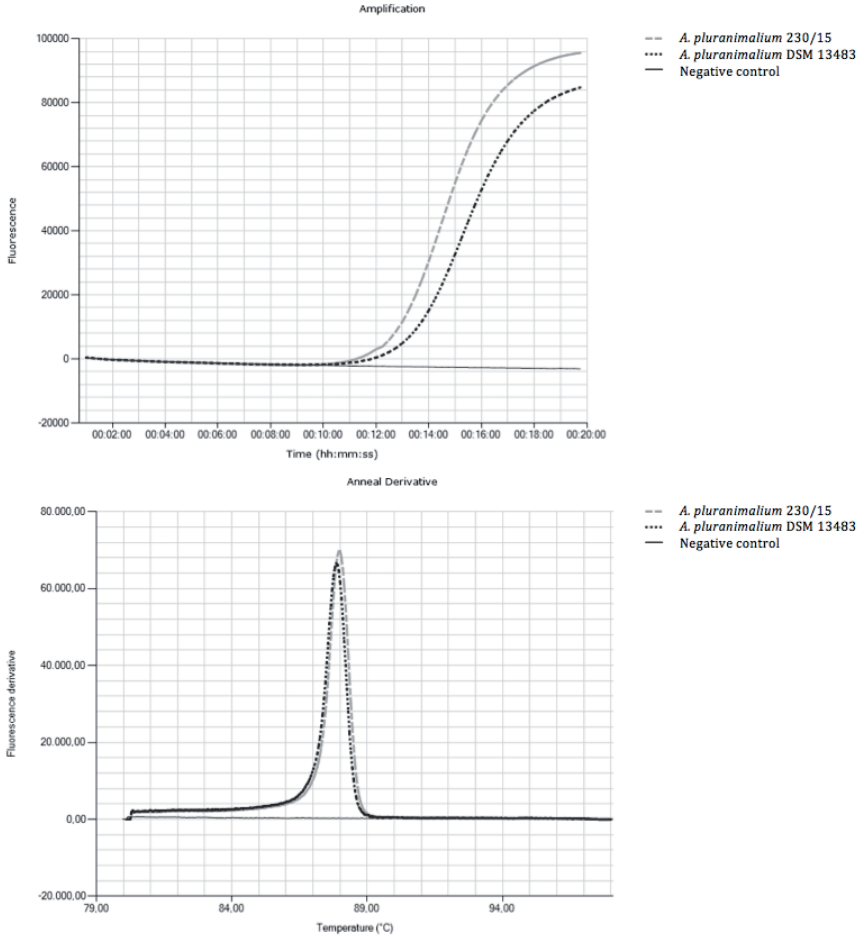

Figure 5. LAMP amplification signal of *A. pluranimalium* 230/15, the type strain *A. pluranimalium* DSM 13483 as positive control as well as a negative control (a). The melting curve (anneal derivation) of the same amplicons (b)

and could be used for molecular identification. Comparable to previous studies (2, 25) this species-specific gene *pla* of *A. pluranimalium* could also be detected successfully by using a *pla* LAMP assay. Comparable LAMP assays have already been used to identify *Leptospira* species (13, 16), *Ersypelothrix rhusiopathiae* (26), *Streptococcus equi* subsp. *zooepidemicus* (12) and ostrich meat (1). These authors described LAMP as a powerfool tool that can be used as an alternative to PCR-based methods since it has a high sensitivity and specificity, a shorter reaction time and a comparably low susceptibility for inhibitors.

The present study gives a first report on the isolation and characterization of *A. pluranimalium* from this origin. However, little is known about the pathogenic importance of the strain.

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Arcanobacterium canis sp. nov., isolated from an otitis externa of a dog and emended description of the genus *Arcanobacterium* Collins et al. 1983 emend. Yassin et al. 2011

Muaz Hijazin[1], Ellen Prenger-Berninghoff[2], Osama Sammra[1], Jörg Alber[1], Christoph Lämmler[1*], Peter Kämpfer[3], Stefanie P. Glaeser[4], Hans-Jürgen Busse[5], Abdulwahed Ahmed Hassan[6], Amir Abdulmawjood[7], Michael Zschöck[8]

[1]Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Giessen, Frankfurter Strasse 107, 35392 Giessen, Germany, [2]Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, Frankfurter Strasse 85-91, 35392 Giessen, Germany, [3]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany, [4] Institute für Mikrobiologie und Molekularbiologie , Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany, [5]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria, [6]De Gezondheidsdienst voor Dieren (Animal Health Service), Postbus 9, 7400 AA Deventer, The Netherlands, [7]Institut für Lebensmittelqualität und –sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany, [8]Landesbetrieb Hessisches Landeslabor, Schubert Strasse 60, 35392 Giessen, Germany.

[*]**Corresponding author:**

Professor Dr. Christoph Lämmler

E-mail: Christoph.Laemmler@vetmed.uni-giessen.de

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P6775[T] is FR846134.

Three supplementary figures and a supplementary table are available with the online version of this paper.

Abstract

A polyphasic taxonomic study was performed on an unidentified *Arcanobacterium*-like Gram-stain-positive bacterium isolated from the otitis externa of a dog. Comparative 16S rRNA gene sequencing showed that the bacterium belonged to the genus *Arcanobacterium* and was most closely related to the type strains *Arcanobacterium haemolyticum* (97.2%), *Arcanobacterium hippocoleae (*96.5%) and *Arcanobacterium phocae* (96.4%). The presence of the major menaquinone MK-9(H_4) supported the affiliation of this strain to genus *Arcanobacterium*. The polar lipid profile contained the major lipids phosphatidylcholine diphosphatidylglycerol, phosphatidylinositol-mannoside and an unidentified phospholipid (PL2). Major fatty acids were $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{18:1}\omega 9c$, and $C_{18:2}\omega 6,9c$/ anteiso-$C_{18:0}$ (detected as a summed feature). $C_{10:0}$ and $C_{12:0}$ were present in minor amounts. The results of physiological and biochemical testing clearly distinguished the unknown bacterium from other species of the genus *Arcanobacterium*. Based on these tests, it is proposed that the unknown bacterium should be classified in the novel species *Arcanobacterium canis* sp. nov. The type strain *Arcanobacterium canis* is P6775^T (CCM 7958^T = CCUG 61573^T = CIP 110339^T). An amended description of the genus Arcanobactium is also provided

Introduction

The genus *Arcanobacterium* was described by Collins *et al.* (1982) and consists of a group of facultative anaerobic, asporogenous Gram-positive rods. According to Yassin *et al.* (2011), this genus was in need of taxonomic revision. These authors proposed that the genus *Arcanobacterium* should be split into two genera, with *Arcanobacterium haemolyticum*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae* constituting the genus *Arcanobacterium* and *Arcanobacterium abortisuis*, *Arcanobacterium bernardiae*, *Arcanobacterium bialowiezense*, *Arcanobacterium bonasi* and *Arcanobacterium pyogenes* being transferred to a new genus, *Trueperella* (Yassin *et al.,* 2011).

During routine microbiological diagnostic testing, a bacterial strain isolated from a dog showed unusual features. Applying a polyphasic taxonomic approach, this strain could be classified within a novel species of genus *Arcanobacterium*.

Strain P6775^T was isolated, together with several other bacterial species (data not shown), from otitis externa of a seven years old, female English Bulldog. The strain was characterized phenotypically including Gram-staining, hemolysis, CAMP-like haemolytic properties and biochemical properties with the help of the API Coryne test system (Biomerieux), by 16S rRNA gene sequencing (Ülbegi-Mohyla *et al.,* 2009; Hassan *et al.,* 2009) and by various other analytical procedures. Phylogenetic analysis was performed using the ARB software package (Ludwig *et al*., 2004) and the corresponding 16S rRNA All-species living tree project (LTP) ARB database release 104, March, 2011 (Yarza *et al*., 2008). The 16S rRNA gene sequence of strain P6775^T was aligned according to the seed alignment in the ARB database. The alignment was corrected manually based on secondary structure information. Phylogenetic trees were reconstructed using the maximum-likelihood method using RAxML 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, the neighbour-joining method using the Jukes-Cantor model (Jukes & Cantor, 1969), and the maximum-parsimony method using DNAPARS version 3.6 (Felsenstein, 2005). Sequence similarities were calculated in ARB based on sequence similarities without the use of an evolutionary substitution model. The 16S rRNA gene sequence of strain P6775^T was a continuous stretch of 1363 bp. Phylogenetic analysis based on 16S rRNA gene sequences spanning *Escherichia coli* positions 128-1379 (Brosius *et al*., 1978). Sequence similarity calculations after a neighbour joint analysis revealed that the strain showed 99.2 % sequence similarity to an uncharacterized *Arcanobacterium* strain, M214/96/1 (GenBank accession number AJ234062). The 16S rRNA gene sequence similarity

of strain P6775^T to *Arcanobacterium* type strains ranged from 95.8 to 97.2% (with *A. haemolyticum* CIP 103370^T as the closest relative) and similarity to *Trueperella* type strains ranged from 93.9 to 94.8%. Phylogenetic tree constructions showed clearly that strain P6775^T clustered within the genus *Arcanobacterium* and was distinct from the genus *Trueperella*. However, the phylogenetic placement within the genus *Arcanobacterium* was not stable when different treeing methods were compared (Fig. 1 and Figures S1-S3, available in IJSEM online). Yassin *et al.* (2011) defined 16S rRNA sequence signature nucleotides that distinguish species of the genera *Arcanobacterium* and *Trueperella*. Strain P6775^T shows all of the signature nucleotides specific for the genus *Arcanobacterium* and was thereby clearly different from *A. hippocoleae* CCUG 44697^T, which shares signature nucleotides with both genera (Yassin *et al*., 2011 and Supplementary Table S1).

Quinones and polar lipids were extracted and analysed according to an integrated procedure reported previously (Tindall, 1990a, b; Altenburger *et al*., 1996). Polyamines were extracted as described previously (Busse & Auling, 1988; Altenburger *et al.*, 1997). For analysis of quinones and polyamines, the HPLC apparatus was used as described by Stolz *et al.* (2007). Fatty acid extraction and analysis was done according to the method described previously (Kämpfer & Kroppenstedt, 1996) using an HP 6890 gas chromatograph with Sherlock MIDI software version 2.11 and the TSBA peak-naming table version 4.1. For fatty acid extraction, the strain was cultured on sheep blood agar at 37 °C for 72h.

The quinone system of strain P6775^T was composed of 73% MK-9 (H_4), 10% MK-9 (H_2), 8% MK-9, 5% MK-8 (H_4), 1% MK-8 (H_2), 1% MK-8, 1% MK-7 (H_2), and 1% MK-7, which is in agreement with the emended description of the genus *Arcanobacterium* (Yassin *et al*., 2011). The polar lipid profile contained the major lipids phosphatidylcholine, diphosphatidylglycerol, phosphatidylinositol-mannoside and an unidentified phospholipid (PL2). Furthermore, moderate amounts of phosphatidylinositol and minor amounts of several unidentified lipids including a glycolipid (GL1), a phosphoglycolipid (PGL1), an aminolipid (AL1) and a phospholipid (PL1) were detected (Fig. 2). The presence of diphosphatidylglycerol and phosphatidylinositol is in accordance with the emended description of *Arcanobacterium* whereas the presence of a phosphoglycolipid and the absence of phosphatidylglycerol are not. The polyamine pattern consisted of 0.17 µmol (g dry weight)$^{-1}$ putrescine, 0.12 µmol (g dry weight)$^{-1}$ spermine and 0.6 µmol (g dry weight)$^{-1}$ spermidine. The typical major fatty acids were $C_{14:0,}$ (16.7%), $C_{16:0,}$ (30.7%) $C_{18:0,}$ (18.2%), and $C_{18:1}\omega 9c$ (19.1%), and $C_{18:2}\omega 6,9c/$ $C_{18:0}$ anteiso (9.9% detected as summed feature). $C_{10:0}$ (2.0%) and $C_{12:0}$ (3.6%) were found in minor

amounts. This fatty acid profile is in congruence with that reported for other *Arcanobacterium* species (Yassin *et al.,* 2011).

The results of physiological characteristics are shown in Table 1 and also in the species description. The results of ApI Coryne tests revealed the profile number 0730363, indicating that strain P6775^T appeared not to be related to the *Arcanobacterium*-like species with ApI Coryne profile number 0130142 isolated from canine otitis externa by Aalbæk *et al*. (2010).

Based on these taxonomic studies, strain P6775^T represents a novel species of the genus *Arcanobacterium*, for which the name *Arcanobacterium canis* sp. nov. is proposed.

Description of *Arcanobacterium canis* sp. nov.

Arcanobacterium canis (can.is. L. gen. n. *canis*, of the dog)

Cells are Gram-strain-positive, oxidase-negative, non-motile, non-spore-forming rods (1-2 μm long and 0.5 μm wide). Growth occurrs on sheep blood agar with a weak zone of haemolysis under microaerobic conditions (the zone of haemolysis is less pronounced under aerobic and anaerobic conditions). CAMP-like reactions occurr with a strain of *Rhodococcus equi* as an indicator strain. Acid is produced from D-glucose, D-ribose, maltose, lactose, saccharose and glycogen, but not from D-xylose or D-mannitol. Activity of the following enzymes is observed: alkaline phosphatase, *β*-glucuronidase, *β*-galactosidase, *α*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase, DNase and amylase. No activity is detected for the following enzymes: nitrate reductase, pyrazinamidase, pyrrolidonyl arylamidase, for aesculin hydrolysis and for serolysis on Loeffler agar, urease, gelatinase, catalase, caseinase and hyaluronidase. The quinone system is composed of the predominant compound menaquinone MK-9 (H_4), moderate amounts of MK-9 (H_2), MK-9 and MK-8 (H_4) and minor amounts of MK-8 (H_2) MK-8, MK-7 (H_2) and MK-7. The polar lipid profile contains the major lipids phosphatidylcholine, diphosphatidylglycerol, phosphatidylinositol-mannoside and an unidentified phospholipid (PL2). Moderate amounts of phosphatidylinositol are detected and minor amounts of several unidentified lipids, including a glycolipid (GL1), a phosphoglycolipid (PGL1), an aminolipid (AL1) and a phospholipid (PL1) are also found. The polyamine pattern contains putrescine, spermine and spermidine. Major fatty acids are $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega9c$, and $C_{18:2}\omega6,9c$/ anteiso-$C_{18:0}$. $C_{10:0}$ and $C_{12:0}$ are present in minor amounts. The 16S rRNA gene sequence of the type strain P6775^T contains all 12 16S rRNA

signature nucleotides defined by Yassin *et al.* (2011) that distinguish species of the genus *Arcanobacterium* from species of the genus *Trueperella*.

The type strain P6775^T **(CCM 7958^T = CCUG 61573^T = CIP 110339^T),** was isolated from canine otitis externa.

Emended description of the genus *Arcanobacterium* Collins *et al.* 1983 emend. Yassin *et al.* 2011.

The description is that of Collins *et al.* (1982) with the emendation of Yassin *et al.* (2011), with the exception that members of the genus may contain a phosphoglycolipid and do not necessarily contain phosphatidylglycerol in their lipid profile.

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Table 1. Physiological characteristics of strain P6775^T and related type strains

Strains: 1, *A. canis* P6775^T; 2, *A. haemolyticum* DSM 20595^T; 3, *A. phocae* DSM 10002^T; 4, *A. pluranimalium* DSM 13483^T; 5, *A. hippocoleae* DSM 15539^T. + Positive reaction; (+), weak reaction; -, negative reaction. Data were obtained in this study after cultivation of the investigated strains for 48 h at 37°C under microaerobic conditions. All strains are negative for nitrate reduction, urease activity and acid production from D-mannitol (API Coryne; bioMerieux) and serolysis on Loeffler agar and positive for acid production from D-glucose and DNase activity (API Coryne).

Phenotypic properties	**1**	**2**	**3**	**4**	**5**
Synergistic CAMP-like reaction*	+	+	+	+	+
Reverse CAMP-reaction	-	+	+	-	-
API Coryne results					
Pyrazinamidase	-	+	-	+	-
Pyrrolidonyl arylamidase‡	-	-	- |(+)|	+	-
Alkaline phosphatase‡	+	+	+	-	+
β-Glucuronidase§	+	-	-	+	+
β-Galactosidase§	+	+	+	- |(+)|	+
α-Glucosidase§	+	+	+	-	+
N-Acetyl-*β*-Glucosaminidase§	+	+	+	-	+
Aesculin hyrdolysis (*β*-Glucosidase)	-	-	-	-	+
Gelatine hydrolysis	-	-	-	+	-
Acid production from (API Coryne):					
D-Ribose	+	+	+	+	-
D-Xylose	-	-	(+)	-	-
Maltose	+	+	+	(+)	+
Lactose	+	+	+	-	+
Saccharose	+	-	+	-	-
Glycogen	+	-	+	-	-
α-Mannosidase‡	+	+	+	+	-
Catalase	-	-	+	+	-
Caseinase	-	-	-	+	-
Amylase	+	-	+	+	-

*Synergistic CAMP-like reactions with staphylococcal β-haemolysin for *A. pluranimalium* and *A. hippocoleae*, with *Streptococcus agalactiae* for *A. haemolyticum* and *A. phocae*, with *Rhodococcus equi* for *A. canis*, *A. haemolyticum*, *A. phocae* and *A. pluranimalium*, with *Psychrobacter phenylpyruvicus* for *A. haemolyticum* and *A. phocae* and *with A. haemolyticum* for *A. pluranimalium* and *A. hippocoleae*.

†Negative or positive reverse CAMP reaction in the zone of staphylococcal *β*-haemolysin.

‡Also tested with tablets containing substrates (Inverness Medical). Results that differ from API Coryne results given in brackets.

§Also tested with 4-methylumbelliferyl-conjugated substrates (Sigma). Results that differ from API Coryne results given in brackets.

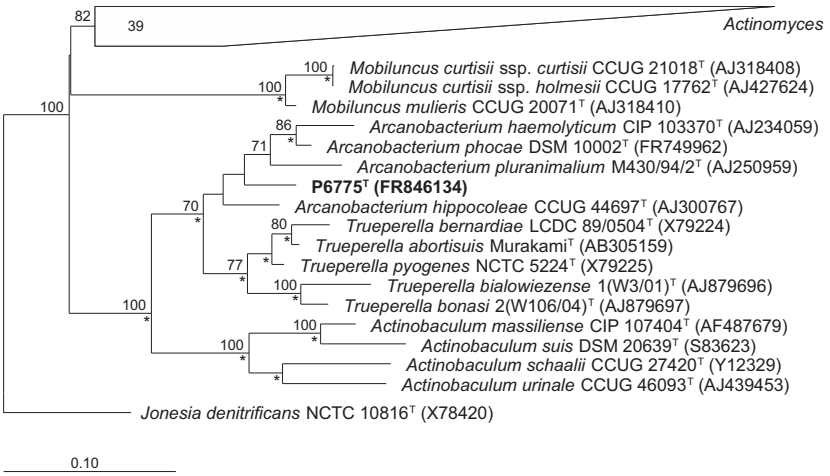


Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strain P6775^T within the *Actinomycetaceae*. *Jonesia denitrificans* NCTC 10816^T was used as an out-group. Numbers at nodes represent bootstrap values ≥70% (100 resamplings). *Escherichia coli* positions 128-1379 (Brosius *et al*., 1978) were considered for analysis. Asterisks mark nodes that were also found in the neighbor-joining and maximum-parsimony trees. Bar, 0.1 substitutions per nucleotide position.

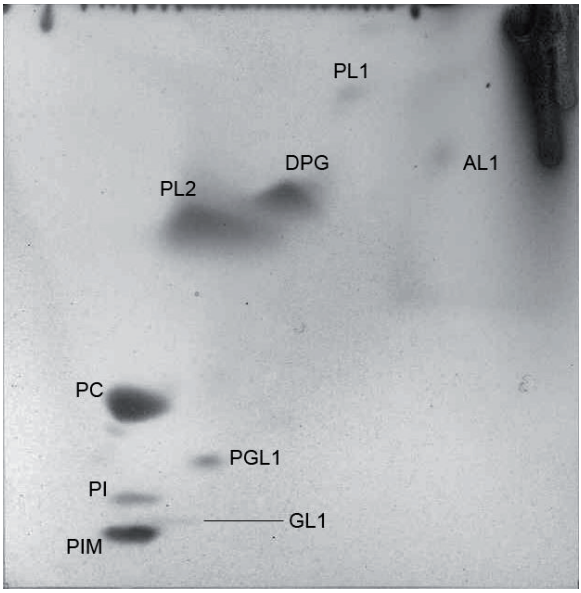
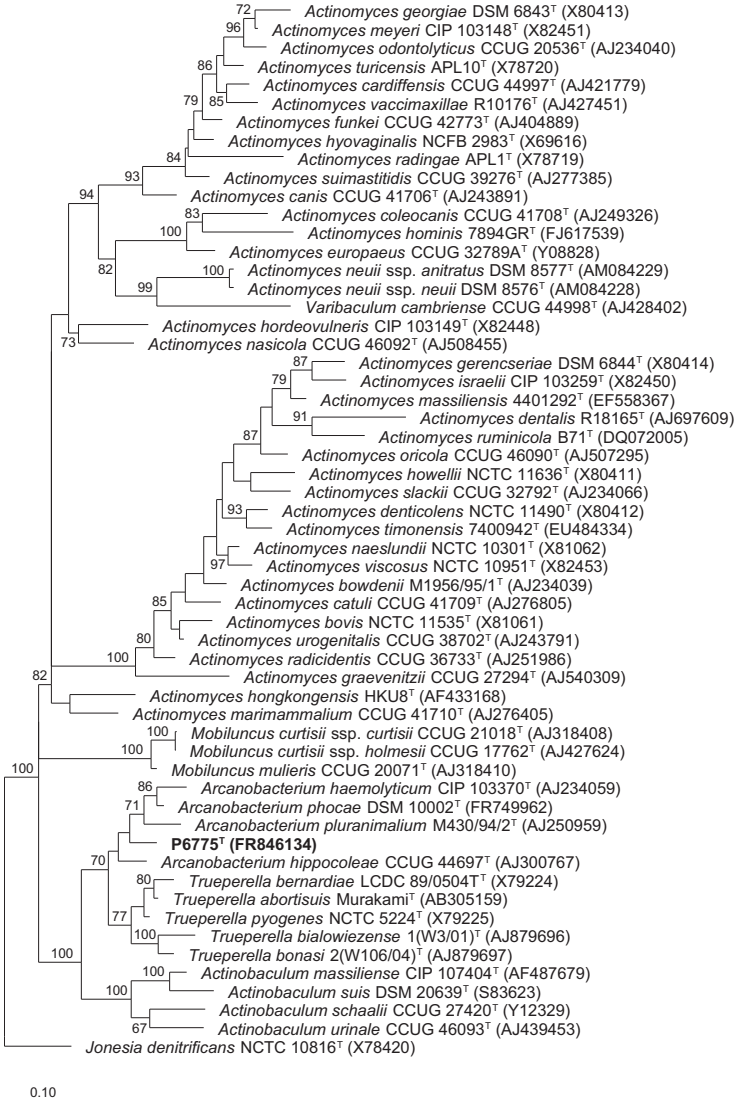


Fig. 2. Total polar lipid profile of strain P6775^T after two dimensional thin layer chromatography and staining with molybdatophosphoric acid. Phosphatidylcholine (PC), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositol-mannoside (PIM), unidentified phospholipid (PL2), unidentified glycolipid (GL1), a phosphoglycolipid (PGL1), aminolipid (AL1), phospholipid (PL1).

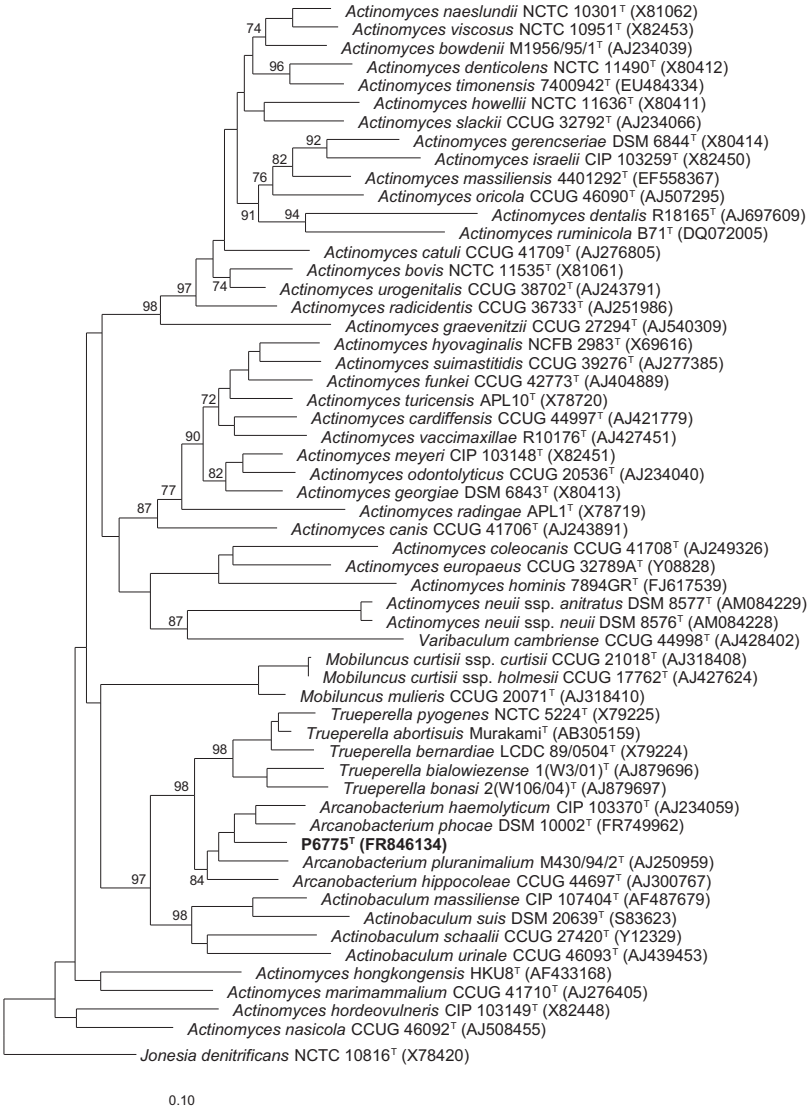
Supplementary Table 1: Determination of 16S rRNA gene signature nucleotides (Yassin *et al*. 2011) for strain P6775^T. Yassin *et al*. (2011) determined twelve signature nucleotides that distinguish species of the genera *Arcanobacterium* and *Trueperella*. 1: P6775^T, 2: genus *Arcanobacterium*, 3: genus *Trueperella*, 4: *A. hippocoleae* CCUG 44697^T that showed signature nucleotides of both genera (as described by Yassin et al., 2011). For positions 440 and 598 signature nucleotides given by Yassin *et al*. (2011) for the genera *Arcanobacterium* and *Trueperella* were corrected according the nucleotide sequences of reference strains obtained in the ARB generated alignment.

Nucleotide position*	1	2	3	4
157-164	**G-U**	G-U	U-G	U-G
440	**U**	*U*	*C*	C
443-491	**C-G**	C-G	U-G	U-G
446-488	**U-G**	U-G	A-U	A-U
492	**U**	U	C	C
497	**U**	U	G	G
598	**U**	*U*	*C*	U
625	**C**	C	U	C
631	**C**	C	U	C
659	**C**	C	U	C
776	**G**	G	U	G
1278	**G**	G	U	G

*Nucleotide position according to *Escherichia coli* (Brosius *et al*., 1978) based on an alignment generated in ARB.



Supplementary Figure S1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strain P6775^T within the *Actinomycetaceae*. *Jonesia denitrificans* NCTC 10816^T was used as out-group. Numbers at nodes represent bootstrap values >70% (100 re-samplings). *Escherichia coli* positions 128 to 1379 (Brosius *et al*., 1978) were considered for analysis. Bar, 0.1 nucleotide substitutions per nucleotide position.



Supplementary Figure S2. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain P6775^T within the *Actinomycetaceae*. *Jonesia denitrificans* NCTC 10816^T was used as out-group. Numbers at nodes represent bootstrap values >70% (100 re-samplings). *E. coli* positions 128 to 1379 (Brosius *et al*., 1978) were considered for analysis. Bar, 0.1 nucleotide substitutions per nucleotide position.

Supplementary Figure S3. Maximum-Parsimony tree based on 16S rRNA gene sequences showing the phylogenetic position of strain P6775^T within the *Actinomycetaceae*. *Jonesia denitrificans* NCTC 10816^T was used as out-group. Numbers at nodes represent bootstrap values >70% (100 re-samplings). *E. coli* positions 128 to 1379 (Brosius *et al*., 1978) were considered for analysis. Bar, 0.1 nucleotide substitutions per nucleotide position.

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Further characteristics of *Arcanobacterium canis*, a novel species of genus *Arcanobacterium*

O. Sammra [a], A. Balbutskaya [a], S. Zhang [b], M. Hijazin [a], S. Nagib [a], C. Lämmler [a,*], A. Abdulmawjood [c], E. Prenger-Berninghoff [d], M. Kostrzewa [e], M. Timke [e]

[a] Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Schubert Str. 81, 35392 Gießen, Germany
[b] Veterinary Pathobiology, Texas A&M University, Texas Veterinary Medical Center, College Station, TX 77843, USA
[c] Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany
[d] Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurterstr. 85-91, 35392 Gießen, Germany
[e] Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany

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ABSTRACT

Comparable to previously conducted phenotypical and genotypical investigations characterizing *Arcanobacterium canis*, a newly described species with the type strain *A. canis* DSM 25104 isolated from an otitis externa of a dog, four additional *A. canis* strains isolated from infections of three dogs and one cat could reliably be identified by phenotypic properties, by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and by sequencing the genomic targets 16S rDNA, 16S-23S rDNA intergenic spacer region, 23S rDNA, and the genes *rpoB* and *gap*. All four *A. canis* investigated in the present study were isolated from the infected animals together with several other bacterial species indicating that the pathogenic importance of *A. canis* remains unclear. However, the detection of peptidic spectra by MALDI-TOF MS and the presented phenotypic and genotypic approaches might help to identify *A. canis* in future and might elucidate the role this species plays in infections of dogs and cats.

1. Introduction

Genus *Arcanobacterium* was described by Collins et al. (1982) as a group of facultative anaerobic, asporogenous Gram-positive rods. However, according to Yassin et al. (2011) this genus was in need of a taxonomic revision. These authors proposed that the genus *Arcanobacterium* should be split into two genera, with *Arcanobacterium haemolyticum*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae* constituting genus *Arcanobacterium* and *Arcanobacterium abortisuis*, *Arcanobacterium bernardiae*, *Arcanobacterium bialowiezense*, *Arcanobacterium bonasi* and *Arcanobacterium pyogenes* being transferred to a new genus *Trueperella*.

However, more recently *Arcanobacterium canis* and *Arcanobacterium phocisimile*, two species which were most closely related to *A. haemolyticum* were described as novel species of genus *Arcanobacterium* (Hijazin et al., 2012b, 2013). The original description of *A. canis* was based on physiological and biochemical characteristics, chemotaxonomic analysis and on 16S rDNA sequencing results of a single strain isolated from an otitis externa of a dog. In the present study this initially described *A. canis* strain and four additionally isolated *A. canis* obtained from three dogs and one cat were identified and further characterized phenotypically, by MALDI-TOF MS fingerprinting and genotypically by amplification and sequencing of 16S rDNA, 16S-23S rDNA intergenic spacer region (ISR), 23S rDNA and by sequencing the target genes encoding beta

* Corresponding author. Tel.: +49 6419938406; fax: +49 6419938409.
E-mail address: Christoph.Laemmler@vetmed.uni-giessen.de (C. Lämmler).

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subunit of RNA polymerase (*rpoB*) and glyceraldehyde 3-phosphate dehydrogenase (*gap*), respectively.

2. Materials and methods

A. canis DSM 25104, further investigated in the present study, was originally isolated from an otitis externa of a dog (Hijazin et al., 2012b). Strains *A. canis* 2308 and *A. canis* P3542 were isolated during routine diagnostic investigations in Giessen, Germany. Strain *A. canis* 2308 was isolated in low numbers together with *Pseudomonas* spp. and *Streptococcus canis* during andrological investigations from semen of a nine year old Doberman. Strain *A. canis* P3542 was isolated together with *Staphylococcus aureus*, beta-hemolytic streptococci, *Bacillus* spp., *Corynebacterium* spp. and yeasts from a paw of an eight year old female English bulldog suffering from otitis. The strains ZT11003002 and ZT12020010 of *A. canis* were isolated together with *Streptococcus canis* from a necrotizing fasciitis of a cat and together with *Escherichia coli* and *Enterococcus* spp. from the peritoneal effusion of a dog, respectively, at the Texas A&M University Veterinary Medical Teaching Hospital, USA. All four newly isolated *A. canis* strains were initially characterized phenotypically and by 16S rDNA sequencing (Hijazin et al., 2012b, 2013; Sammra et al., 2013). Reference strains used for comparative purposes belonged to various species of genus *Arcanobacterium* (Sammra et al., 2013; Hijazin et al., 2013). The sample preparation and analysis and data processing for MALDI-TOF MS were performed as described previously (Hijazin et al., 2012a). The microbial identification by MALDI-TOF MS was based on matching the unknown spectra against the main spectra of the reference strain and the database resulting in log (score) values ranging from 0 (no homology) to 3 (absolute identity).

Sequencing of ISR, 23S rDNA and the genes *rpoB* and *gap* were performed with the oligonucleotide primers described (Ülbegi-Mohyla et al., 2010a,b; Sammra et al., 2013).

3. Results and discussion

The name of the novel species *A. canis* was given because the initially characterized strain was isolated from an otitis externa of a dog. However, *A. canis* ZT11003002 of the present study was isolated from a necrotizing fasciitis of a cat. All four strains newly characterized in the present study could reliably be identified as *A. canis* by phenotypic

Table 1
Phenotypical properties of four *A. canis* strains investigated in the present study and *A. canis* DSM 25104.

Phenotypic properties	*A. canis* 2308	*A. canis* P3542	*A. canis* ZT11003002	*A. canis* ZT12020010	*A. canis* DSM 25104**
Hemolysis on sheep blood agar	+	+	+	+	+
Hemolysis on rabbit blood agar	+(+)	+(+)	+(+)	+(+)	+(+)
CAMP-like reaction with:*					
Streptococcus agalactiae	–	–	–	–	–
Rhodococcus equi	+	+	+	+	+
Reverse CAMP reaction	–	–	–	–	–
Nitrate reduction	–[1]	–[1]	–[1]	–[1]	–[1]
Pyrazinamidase	–[1]	–[1]	–[1]	–[1]	–[1]
Pyrrolidonyl arylamidase	+[1]	(+)[1]	+[1]	(+)[1]	–[1]
Alkaline phosphatase	+[1]	+[1]	+[1]	+[1]	+[1]
β-Glucuronidase (β-GUR)	(+)[1],+[2,3]	(+)[1],+[2,3]	+[1,2,3]	+[1,2,3]	+[1,2,3]
α-Galactosidase (α-GAL)	+[2]	+[2]	+[2]	+[2]	+[2]
β-Galactosidase (β-GAL)	(+)[1], +[3]	(+)[1], +[3]	+[1,3]	(+)[1], +[3]	+[1,3]
α-Glucosidase (α-GLU)	+[1,2,3]	+[1,2,3]	+[1,2,3]	+[1,2,3]	+[1,3]
β-Glucosidase (β-GLU)	–[2]	–[2]	–[2]	–[2]	–[2]
N-acetyl-β-Glucosaminidase (β-NAG)	+[1,3]	+[1,3]	+[1,3]	+[1,3]	+[1,3]
Esculin (β-Glucosidase)	–[1]	–[1]	–[1]	–[1]	–[1]
Urease	–[1]	–[1]	–[1]	–[1]	–[1]
Hydrolysis of Gelatine	–[1]	–[1]	–[1]	–[1]	–[1]
Acid formation from:					
D-Glucose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Ribose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Xylose	–[1]	–[1]	–[1]	–[1]	–[1]
D-Mannitol	–[1]	–[1]	–[1]	–[1]	–[1]
D-Maltose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Lactose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Saccharose	+[1]	+[1]	+[1]	+[1]	+[1]
Glycogen	–[1]	+[1]	+[1]	+[1]	+[1]
α-Mannosidase	–[2]	+[2]	+[2]	+[2]	+[2]
Catalase	–	–	–	–	–
Serolysis on Loeffler agar	–	–	–	–	–
Caseinase	–	–	–	–	–
DNase	+	+	+	+	+
Starch hydrolysis	–	+	–	+	+

The reactions are shown as follows: * = synergistic CAMP-like reaction with indicator strains; ** = results obtained from Hijazin et al., 2012b; +(+), slightly enhanced positive reaction; +positive reaction; (+), weak positive reaction; –, negative reaction 1 = Api-Coryne test system (bioMérieux, Nürtingen, Germany); 2 = tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); 3 = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

properties and by 16S rRNA sequencing. The phenotypic properties appeared to be almost identical to previously characterized *A. canis* DSM 25104. Compared to the type strain *A. canis* DSM 25104, phenotypic differences were observed in four strains for the enzyme pyrrolidonyl arylamidase, in one strain for glycogen fermentation and the enzyme α-mannosidase and in two strains for starch hydrolysis, respectively (Table 1).

As mentioned by numerous authors MALDI-TOF MS appeared to be a powerful tool for species identification of a broad spectrum of bacteria including Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011). This technique had previously been successfully used for rapid and reliable identification of bacteria of genera *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012a). Using the current Bruker database the MALDI-TOF MS analysis of the *A. canis* strains of the present study did not allow an identification of this hitherto unknown species to species level. However, using the MALDI Biotyper 3.1 software package the log (score) value of all four *A. canis* investigated in the present study matched against *A. canis* DSM 25104 with log score values between 2.4 and 2.7 indicating that all four strains could also be classified to this newly discovered species. The hierarchical cluster analysis formed a distinct cluster separated from other species of genus *Arcanobacterium* indicating that *A. canis* is representing a novel species of genus *Arcanobacterium*. Inclusion of *A. canis* into the reference database will allow the detection of this new species by MALDI-TOF MS in future. A typical dendrogram of the MALDI-TOF results of the four newly described *A. canis* of the present study, *A. canis* DSM 25104 and various other species of genus *Arcanobacterium* is shown in Fig. 1.

The species classification by 16S rDNA sequencing revealed a ≥ 99.9% sequence identity of all four *A. canis* strains to *A. canis* DSM 25104 (Fig. 2). All four *A. canis* together with *A. canis* DSM 25104 could additionally be identified by amplification and sequencing of ISR (GenBank accession numbers HF947291, HF947292, HF947293, HF947294, HE616814) and 23S rDNA (HF947295, HF947296, HF947297, HF947298, HE616813)

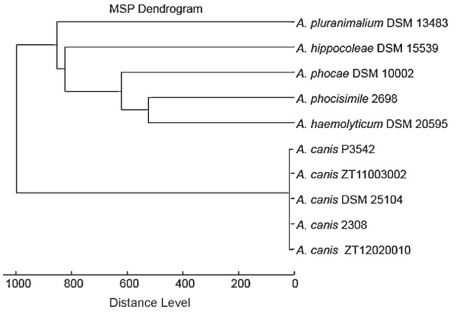

Fig. 1. MALDI-TOF MS spectra of the *A. canis* strains of the present study, reference strain *A. canis* DSM 25104 and various other species of genus *Arcanobacterium*.

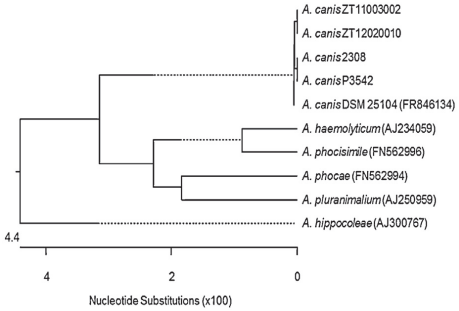

Fig. 2. Dendrogram analysis of 16S rDNA sequences of the *A. canis* strains of the present study and various reference strains of genus *Arcanobacterium* obtained from NCBI GenBank.

and the target genes *rpoB* (HF947302, HF947303, HF947300, HF947301, HF947299) and *gap* (HF947281, HF947282, HF947283, HF947284, HF947280) yielding for all four molecular targets an identity of ≥99.5% of all five *A. canis* strains among each other, respectively, and a clear separation of *A. canis* from other species of genus *Arcanobacterium*. The molecular targets ISR, 23S rDNA and *rpoB* had already been used for genotypic characterization of bacteria of genus *Arcanobacterium* and *Trueperella* (Ülbegi-Mohyla et al., 2010a,b), gene *gap* as novel target to identify an *A. haemolyticum* strain isolated from a donkey (Sammra et al., 2013). A typical dendrogram using the sequencing results of the novel target gene *gap* is shown in Fig. 3.

According to the results of the present study the species identity of *A. canis* as novel species of genus *Arcanobacterium* could reliably be determined by phenotypic properties, by MALDI-TOF MS and by sequencing various genomic targets. The usefulness of the determination of peptidic spectra and the various genotypical targets for identification of this species has to be further investigated with additional *A. canis* isolated from dog and cat, possibly other animals or humans. All hitherto described *A. canis* were isolated from mixed infections with several other bacteria indicating that the pathogenic

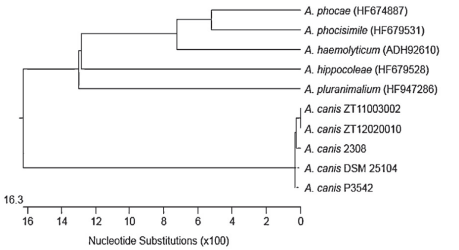

Fig. 3. Dendrogram analysis of sequences of gene *gap* of the *A. canis* strains of the present study and various reference strains of genus *Arcanobacterium* obtained from NCBI GenBank.

importance of this novel bacterial species remains to be elucidated.

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Arcanobacterium phocisimile sp. nov., isolated from harbour seals

Muaz Hijazin[1], Osama Sammra[1], Hivda Ülbegi[1], Samy Nagib[1], Jörg Alber[1], Christoph Lämmler[1*], Peter Kämpfer[2], Stefanie P. Glaeser[2], Hans-Jürgen Busse[3], Johannes Kassmannhuber[3], Ellen Prenger-Berninghoff[4], Reinhard Weiss[4], Ursula Siebert[5], Abdulwahed Ahmed Hassan[6], Amir Abdulmawjood[7] and Michael Zschöck[8]

[1]Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany, [2]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany, [3]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria, [4]Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany, [5]Forschungs- und Technologiezentrum Westküste, Zentrale Einrichtung der Christian-Albrechts-Universität zu Kiel, 25761 Büsum, Germany, [6]De Gezondheidsdienst voor Dieren (Animal Health Service), 7400 AA Deventer, The Netherlands, [7]Institut für Lebensmittelqualität und –sicherheit, Stiftung Tierärztliche Hochschule Hannover, 30173 Hannover, Germany, [8]Landesbetrieb Hessisches Landeslabor, 35392 Giessen, Germany.

Running title: *Arcanobacterium phocisimile* sp. nov.
Corresponding author: Professor Dr. Christoph Lämmler
Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Giessen
Frankfurter Straße 107, D-35392 Giessen, Germany
Tel.: +49-6419938406, Fax: +49-6419938409
E-mail: Christoph.Laemmler@vetmed.uni-giessen.de

Abbreviations: BOX-PCR, BOX-A1R-based repetitive extragenic palindromic PCT; ERIC-PCT, enterobacterial repetitive intergenic consensus PCR; RAPD, random amplification polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 2698^T and 4112 are FN562996 and FN562998, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

Abstract

A polyphasic taxonomic study was performed on two previously unidentified *Arcanobacterium*-like Gram-positive strains isolated from harbor seals. Comparative 16S rRNA gene sequencing showed that both bacteria belonged to the genus *Arcanboacterium* and were most closely related to *Arcanobacterium haemolyticum* CIP 103370^T (98.4% 16S rRNA gene sequence similarity), *A. canis* P6775^T (97.4%), A. phocae DSM 10002^T (97.4%), *A. pluranimalium* M430/94/2^T (95.7%) and *A. hippocoleae* CCUG 44697^T (95.5%). The presence of the major menaquinone MK-9 (H_4) supported the affiliation of the isolates with the genus *Arcanobacterium*. The polar lipid profile consisted of major amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, an unidentified phospholipid and two unidentified glycolipids. The major fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega9c$ and summed feature 5 (comprising $C_{18:2}\omega6,9c$ and /or anteiso-$C_{18:0}$). Physiological and biochemical tests clearly distinguished the isolates from other members of the genus *Arcanobacterium*. Based on the common origin and various physiological properties comparable to those of *A. phocae*, it is proposed that the isolates are classified as members of a novel species with the name *Arcanobacterium phocisimile* sp. nov. The type strain is 2698^T (=LMG 27073^T; = CCM 8430^T).

Introduction

The genus *Arcanobacterium* was described by Collins *et al.* (1982) as a group of facultative anaerobic, asporogenous Gram-positive rods. However, according to Yassin *et al.* (2011) this genus was in need of a taxonomic revision as indicated from the phylogenetic split of the genus into two branches that is supported by different quinone systems. These authors proposed that the genus *Arcanobacterium* should be split into two genera, with *Arcanobacterium haemolyticum*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae* constituting the genus *Arcanobacterium sensu stricto* and *Arcanobacterium abortisuis*, *Arcanobacterium bernardiae*, *Arcanobacterium bialowiezense*, *Arcanobacterium bonasi* and *Arcanobacterium pyogenes* being transferred to a new genus, *Trueperella* (Yassin *et al.,*2011). More recently, *Arcanobacterium canis* could be described as novel species of this genus (Hijazin *et al*., 2012).

During routine microbiological diagnostics two strains isolated from specimens of harbour seals showed unusual features. Applying a polyphasic taxonomic approach these two strains could be classified as novel species of genus *Arcanobacterium* for which the name *Arcanobacterium phocisimile* sp. nov. is proposed. Both strains were isolated together with several other bacterial strains (data not shown) during a monitoring program from free living harbour seals of the German North Sea. Strain 2698^T was recovered in 2004 from a vaginal swab of a harbour seal with a length of 130 cm and a weight of 31 kg, strain 4112 in 2007 from an anal swab of a male harbour seal with a length of 185 cm and a weight of 93 kg. Both strains were characterized phenotypically investigating hemolytic properties, CAMP-like hemolytic properties and biochemical properties with the help of API Coryne test system (Biomerieux, Nürtingen, Germany), by 16S rRNA gene sequencing (Ülbegi-Mohyla *et al.,* 2009; Hassan *et al.,* 2009) and by various other analytical procedures (Hijazin *et al*., 2012).

Phylogenetic analysis based on the nearly full-length 16S rRNA gene sequence was performed in ARB release 5.2 (Ludwig *et al*., 2004) using the All-Species Living Tree Project (LTP; Yarza *et al*., 2008) ARB database release LTPs106 (August 2011). Sequences not included in the database were aligned with SINA (v1.2.9) according to the SILVA seed alignment [http://www.arb-silva.de; Pruesse *et al*. (2007)] and implemented in the database. The alignment was checked manually based on secondary structure information. Pairwise sequence similarities were calculated in ARB without the use of an evolutionary substitution model. Phylogenetic trees were constructed with the Maximum likelihood method using RAxML v7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis and the neighbour-joining method (Saitou & Nei, 1987) with the Jukes-Cantor correction (Jukes &

Cantor, 1969). Both phylogenetic trees were calculated with 100 resamplings (Bootstrap analysis; Felsenstein, 1985) and based on 16S rRNA gene sequences between positions 128 to 1379 according to *Escherichia coli* numbering, Brosius *et al*. (1978). The 16S rRNA gene sequences of the strains 2698^T and 4112 were a continuous stretch of 1337 and 1339 bp, respectively. Strains 2698^T and 4112 shared identical 16S rRNA gene sequences and shared 95.5% to 98.4 % sequence similarities to *Arcanobacterium* type strains (with *A. haemolyticum* as closest relative) and to *Trueperella* type strains 93.2 % to 94.1 %. Sequence similarities to type strains of all other genera of the family *Actinomycetaceae* were below 91.5%. Phylogenetic tree construction clearly showed that both strains clustered within the genus *Arcanobacterium* and were distinguished from the genus *Trueperella*. However, the clustering within the genus *Arcanobacterium* was not stable and varied between the different used treeing methods (Figure 1 and Supplementary Fig. 1).

Previously, 12 signature nucleotides of the 16S rRNA gene were defined by Yassin *et al*. (2011) to distinguish the genera *Arcanobacterium* and *Trueperella.* Strains 2698^T and 4112 had all signature nucleotides specific for the genus *Arcanobacterium* (subcluster I, Yassin *et al*., 2011) and could therefore be clearly differentiated from the genus *Trueperella* (Table S1).

For further genotypic differentiation of strains 2698^T and 4112 among each other and with their closest neightbour, *A. haemolyticum* DSM 20595^T, this study used DNA-DNA hybridization (Ziemke *et al*. 1998) and three genomic fingerprinting methods: the repetitive element-primed PCRs BOX-A1R-based repetitive extragenic palindromic PCR (BOX-PCR) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), and random amplification polymorphic DNA (RAPD) analysis. Reactions were performed in a total volume of 15 µL containing 60 ng genomic DNA, 1 × DreamTaq Buffer, 0.2 mM each dNTPs, 1.0 µM each primer and 0.8 U DreamTaq™ DNA polymerase (Fermentas). Primers ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC-3′) were used for ERIC-PCR (Versalovic *et al*., 1991). Primer BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) was used for BOX-PCR (Versalovic *et al.*, 1994). Primer A (5'-CTG GCG GCT TG-3′) (Ziemke *et al.*, 1997) was used for RAPD-PCR. Reaction conditions for ERIC and BOX PCRs were 95°C for 3 min, 30 cycles of 94°C for 30 sec, 53°C for 1 min, and 70°C for 8 min and 70°C for 16 min. Reactions conditions for RAPD-PCR were 95°C for 3 min, 45 cycles of 95°C for 15 sec, 34°C for 1 min, 72°C for 2 min, and 72°C for 10 min. All reactions were performed in a MyCycler (Bio-Rad). Amplification products were separated on 1.5 % agarose gels in 1 × TBE puffer for 2.5

hours at 5.1 V cm^{-1}, strained with ethidiom bromide and documented using a Fluor-S MultiImager (Bio-Rad).

DNA-DNA relatedness between strains 2698^T and 4112 was 82.5% (reciprocal value 99.0%) and between the isolates and A. haemolyticum DSM 20595^T were 59.1% (66.5%) and 64.7% (49.1%), respectively, which indicated that strains 2698^T and 4112 represent two strains a single species distinct from *A. haemolyticum.* This finding was supported by the genomic fingerprint pattern, which showed slight differences between strain 2698^T and strain 4112 but many differences between the isolates and *A. haemolyticum* DSM 20595^T (Fig. 2).

Quinones and polar lipids of strain 2698^T were extracted and analyzed according to an integrated procedure (Tindall, 1990a, b; Altenburger *et al*., 1996). For analysis of quinones, the HPLC apparatus described by Stolz *et al*. (2007) was used. Fatty acid extraction and analysis was done according to the method described previously (Kämpfer & Kroppenstedt, 1996) using a HP 6890 gas chromatograph with a Sherlock MIDI software version 2.11 and TSBA peak-naming table version 4.1. The isolate was cultured on sheep blood agar (Oxoid) at 37° C for 72h without agitation before fatty acid extraction.

The respiratory quinones of strain 2698^T were MK-9 (H_4) (90%), MK-9 (6%), MK-9 (H_2), and (1%) and MK-8 (H_4) (1%), which is in line with the emended description of genus *Arcanobacterium* (Yassin *et al*., 2011). The polar lipid profile consists of major amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol-mannosit, two unidentified glycolipids (GL2, GL3) and an unidentified phospholipid (PL3) and moderate amounts of two unidentified glycolipids (GL1, GL4, GL5), two unidentified phospholipids (PL1, PL2), one unidentified aminolipid and three further unidentified lipids (Fig. 3). The presence of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol is in accordance with the emended description of *Arcanobacterium*. Analysis of the type strain of *Arcanobacterium haemolyticum* showed that phosphatidylinositol mannoside, GL2, GL4 PL1 and PL2 were present (data not shown) though the emended genus description does not list the absence of any phosphoglycolipid. On the other hand, some of the phospholipids reported for *A. haemolyticum* could not detected in this study. Interestingly GL2 and GL3 could only be detected in strain 2698^T and not for strain 4112. Because of the differences in the polar lipid profiles of strains 2698^T and 4112 and of *A. haemolyticum* obtained by Yassin *et al*. (2011) and this study, caution is needed in drawing conclusions based on polar lipid profiles on the taxonomy of the genus

Arcanobacterium. The major fatty acids of strain 2698^T were $C_{16:0}$, (20.7%), $C_{18:0}$ (27.1%), $C_{18:1}\omega 9c$ (25.4%) and summed feature (comprising $C_{18:2}\omega 6,9c$ and/or anteiso-$C_{18:0}$; 21.3%), and minor amounts of $C_{10:0}$ (1.2%), $C_{12:0}$ (1.03%), $C_{14:0}$, (1.0%), $C_{17:0}$ (0.9%) and anteiso-$C_{17:0}$ (1.4%) were also found. Similar results were obtained for strain 4112: major fatty acids $C_{16:0}$, (19.5%), $C_{18:0}$ (23.7%), $C_{18:1}\omega 9c$ (26.6%) and summed feature 5 (comprising $C_{18:2}\omega 6,9c$ and/or anteiso-$C_{18:0}$; 22.2%) and minor amounts of $C_{10:0}$ (0.9%), $C_{12:0}$ (0.9%), $C_{14:0}$, (0.9%), $C_{17:0}$ (1.4%) and anteiso-$C_{17:0}$ (0.8%) as well as $C_{18:1}\omega 6c$ (1.7%). These fatty acid compositions are congruent with those reported for other members of the genus *Arcanobacterium* (Yassin *et al.,* 2011, Hijazin *et al,* 2012).

The results of physiological characteristics are given in Table 1 and the species description. Based on these taxonomic studies, the isolates represent two strains of a novel species of the genus *Arcanobacterium*. Because harbour seals are the origin of *A. phocae* and because *A. phocae* and the two strains have similar CAMP-like hemolytic properties and biochemical properties, the name *A. phocisimile* sp. nov. is proposed.

Description of *Arcanobacterium phocisimile* sp. nov.

Arcanobacterium phocisimile (pho.ci.si'mi.le. L. gen. n. *phocae* of a seal and also a bacterial epithet (*Arcanobacterium phocae*); L. neut. adj. *simile* like, resembling, similar; N.L. neut. adj. *phocisimile* resembling (*Arcanobacterium*) *phocae*.

Cells are non-motile, non-spore-forming, Gram-positive and oxidase-negative rods (1-2 μm long and 0.5 μm wide). Grows on sheep blood agar, with a weak zone of hemolysis under microaerobic conditions and a less pronounced zone under aerobic and anaerobic conditions. CAMP-like reactions occurr with various indicator strains and a reverse CAMP is observed. Acid is produced from D-glucose, D-ribose, maltose, lactose, saccharose and glycogen, but not from D-xylose or D-mannitol. Positive for pyrazinamidase, *β*-galactosidase, *α*-glucosidase, *α*-mannosidase, catalase, DNase and amylase, but negative for nitrate reductase, *β*-glucuronidase, *α*-galactosidase, *β*-glucosidase, for hippurate, for esculin hydrolysis, urease, gelatinase, serolysis on Loeffler agar and caseinase. Variable results using different test systems were observed for pyrrolidonyl arylamidase, alkaline phosphatase and *N*-acetyl-*β*-glucosaminidase. The quinone system is composed of the predominant compound MK-9 (H_4), minor amounts or MK-9 and trace amounts of MK-9 (H_2) and MK-8 (H_4). The polar lipid

profile contains major amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosidase, two unidentified glycolipids and an unidentified phospholipid and moderate amounts of three unidentified glycolipids, two unidentified phospholipids, an unidentified aminolipid and three further unidentified lipids. The major fatty acids are $C_{18:0}$, $C_{18:1}\omega 9c$, $C_{16:0}$, and summed feature 5 (comprising $C_{18:2}\omega 6,9c$ and/or anteiso-$C_{18:0}$).

The type strain 2698^T (=LMG 27073^T = CCM 8430^T), was isolated from a vaginal swab of a harbour seal. The 16S rRNA gene sequence of the type strain contains all 12 16S rRNA gene signature nucleotides defined by Yassin *et al.* (2011) that distinguish species of the genus *Arcanobacterium* from species of the genus *Trueperella*.

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Table 1. Physiological characteristics of strain 2689^T and 4112 and members of the genus *Arcanobacterium*

Strains: 1, *Arcanobacterium phocisimile* 2698^T; 2, *Arcanobacterium phocisimile* 4112; 3, *Arcanobacterium canis* CIP 110339^T; 4, *Arcanobacterium haemolyticum* DSM 20595^T; 5, *Arcanobacterium hippocoleae* DSM 15539^T; 6, *Arcanobacterium phocae* DSM 10002^T; 7, *Arcanobacterium .pluranimalium* DSM 13483^T. Data were obtained in this study after cultivation for 48 h at 37°C under microaerobic conditions. All strains are positive to DNase and fermentation of D-glucose and maltose. All strains are negative for urease, serolysis on Loeffler agar and fermentation of D-mannitol. +, Positive; w, weakly positive; -, negative.

Phenotypic properties	1	2	3	4	5	6	7
Synergistic CAMP-like reaction							
Staphylococcal *β*-haemolysin	-	-	-	-	+	-	+
Strepotococcus agalactiae	+	+	-	+	-	+	-
Rhodococcus equi	+	+	+	+	-	+	+
Psychrobacter phenylpyruvicus	+	+	-	+	-	+	-
A. haemolyticum	-	-	-	-	+	-	+
Reverse CAMP-reaction							
Staphylococcal *β*-haemolysin	+	+	-	+	-	+	-
Nitrate reduction*	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]
Pyrazinamidase*	+	+	-	+	-	-	+
Pyrrolidonyl arylamidase*†	- (+)	- (+)	- (-)	- (-)	- (-)	- (+)	- (+)
Alkaline phosphatase*‡	w (-)	w (-)	+ (+)	+ (+)	+ (+)	+ (+)	- (-)
β-Glucuronidase*‡	- (-)	- (-)	+ (+)	- (-)	+ (+)	- (-)	+ (+)
β-Galactosidase*‡	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	- (w)
α-Glucosidase*‡	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	- (-)
N-Acetyl-*β*-Glucosaminidase*‡	- (+)	- (+)	+ (+)	+ (+)	+ (+)	+ (+)	- (-)
Esculin (*β*-Glucosidase)	-	-	-	-	w	-	+
Gelatine*	-	-	-	-	-	-	+
Fermentation of:*							
D-Ribose	+	+	+	+	-	+	+
D-Xylose	-	-	-	-	-	w	-
Lactose	+	+	+	+	+	+	-
Saccharose	+	+	+	-	-	+	-
Glycogen	+	+	+	-	-	+	-
α-Mannosidase†	+	+	+	+	-	+	+
Catalase	+	+	-	-	-	+	+
Caseinase	-	-	-	-	-	-	+
Amylase	+	+	+	-	+	+	+

* Data were obtained using API Coryne (bioMérieux)

† Data in parentheses were obtained using substrate-containing tablets (Inverness Medical).

‡ Data in parenthesis were obtained using 4-methylumbelliferyl-conjugated substrates (Sigma).

Figures

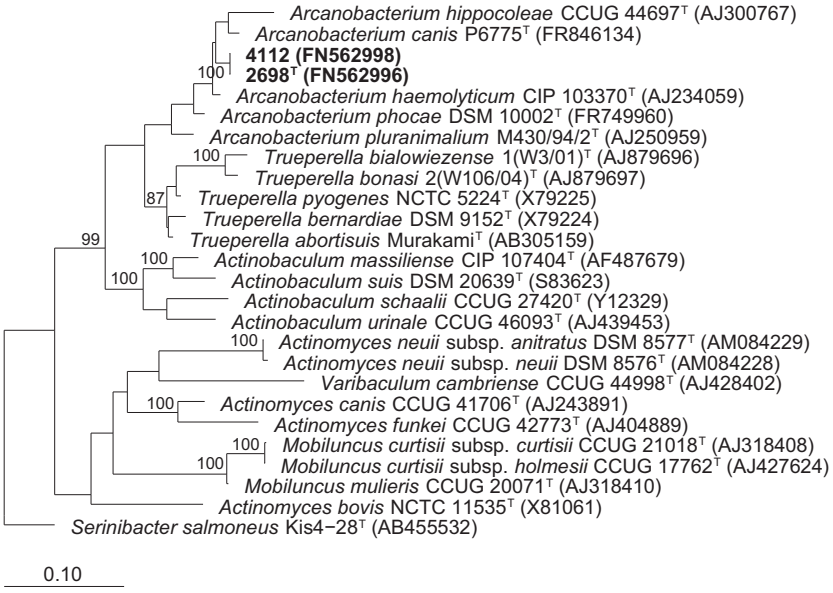

Fig. 1. Maximum-likelihood tree showing the phylogenetic relationships of strains 2698^T and 4112 with members of the genera *Arcanobacterium* and *Trueperella* and some members of the family *Actinomycetaceae*. Bootstrap values (>70%) based on 100 reamplicates are shown at branch nodes. *Serinibacter salmoneus* Kis4-28^T was used as an outgroup. Bar, 0.10 substitutions per nucleotide

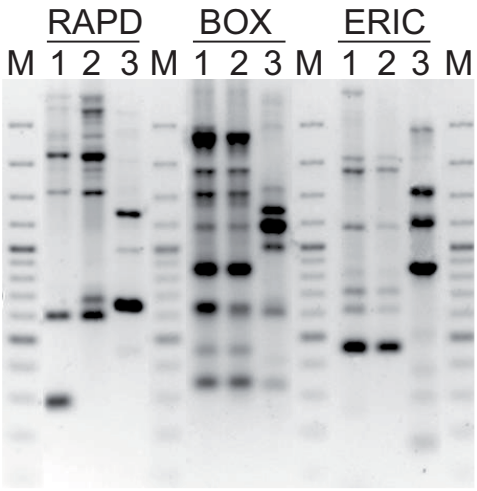

Fig. 2. Genomic fingerprint patterns generated with RAPD, BOX, and ERIC-PCR. Strains: 1, *Arcanobacterium phocisimile* sp. nov. 2698^{T}; 2, *Arcanobacterium phocisimile* sp. nov. 4112; 3, *A. haemolyticum* DSM 20595^{T}. M., GeneRuler100bp Plus DNA Ladder (Fermentas).

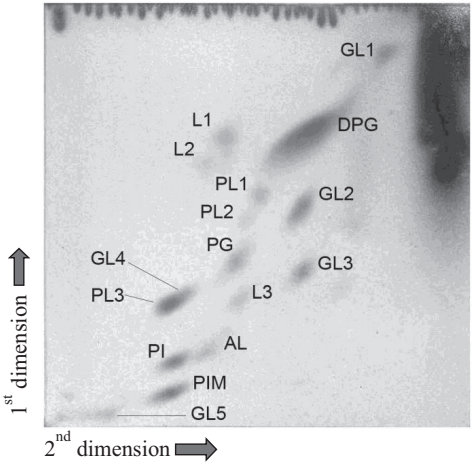

Fig. 3. Total polar lipid profile of strain 2698^T after two dimensional TLC and staining with molybdatophosphoric acid. AL, Aminolipid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; L, polar lipid; P, phospholipid; PG, phosphatidylglycerol; Pl, phosphatidylinositol; PIM, phosphatidylinositol mannoside.

Supplementary Table 1: Assignment of 16S rRNA gene signature nucleotides for strains 2698^T and 4112. The twelve listed signature nucleotides were determined by Yassin *et al*. (2011) to distinguish species of the genera *Arcanobacterium* and *Trueperella*. Groups: 1: 2698^T and 4112, 2: genus *Arcanobacterium* (Subcluster I, Yassin *et al*., 2011), 3: genus *Trueperella* (Subcluster II, Yassin *et al*., 2011), 4: *A. hippocoleae* CCUG 44697^T, 5: *A. canis* P6775^T. *Arcanobacterium hippocoleae* CCUG 44697^T and *A. canis* P6775^T showed signature nucleotides of both genera (as described by Yassin *et al*., 2011, Hijazin *et al*., 2012). For positions 157-164, 440, and 598 signature nucleotides given by Yassin *et al*. (2011) for the genera *Arcanobacterium* and *Trueperella* were corrected according the nucleotide sequences of reference strains obtained in the ARB generated alignment as described previously (Hijazin *et al.,* 2012).

Nucleotide position*	1	2	3	4	5
157-164	U-G	U-G	G-U	U-G	**G-U**
440	U	U	C	C	**U**
443-491	C-G	C-G	U-G	U-G	**C-G**
446-488	U-G	U-G	A-U	A-U	**U-G**
492	U	U	C	C	**U**
497	U	U	G	G	**U**
598	U	*U*	*C*	U	**U**
625	C	C	U	C	**C**
631	C	C	U	C	**C**
659	C	C	U	C	**C**
776	G	G	U	G	**G**
1278	G	G	U	G	**G**

*Nucleotide position according to *E. coli* (Brosius *et al*., 1978) based on the alignment performed in ARB.

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Research Article

Further Studies on *Arcanobacterium phocisimile*: a Novel Species of Genus *Arcanobacterium*

Osama Sammra,[1] Anna Balbutskaya,[1] Muaz Hijazin,[1] Samy Nagib,[1] Jörg Alber,[1] Christoph Lämmler,[1] Amir Abdulmawjood,[2] Ellen Prenger-Berninghoff,[3] Markus Timke,[4] Markus Kostrzewa,[4] and Ursula Siebert[5]

[1] *Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, Schubertstraße 81, 35392 Gießen, Germany*
[2] *Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany*
[3] *Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurterstraße 85-91, 35392 Gießen, Germany*
[4] *Entwicklung Bioanalyse, Bruker Daltonik GmbH, Fahrenheitstraße 4, 28359 Bremen, Germany*
[5] *Institut für Terrestrische und Aquatische Wildtierforschung, Stiftung Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany*

Correspondence should be addressed to Christoph Lämmler; christoph.laemmler@vetmed.uni-giessen.de

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Arcanobacterium phocisimile, a newly described species with the type strain *A. phocisimile* 2698^T isolated from a vaginal swab of a harbour seal and four additional *A. phocisimile* strains also isolated from four harbour seals could reliably be identified by phenotypic properties, by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), and by sequencing the genomic targets 16S rDNA and 16S-23S rDNA intergenic spacer region and the genes *rpoB* and *gap*. The *A. phocisimile* strains investigated in the present study were isolated together with several other bacterial species indicating that the pathogenic importance of *A. phocisimile* remains unclear. However, the detection of peptidic spectra by MALDI-TOF MS and the presented phenotypic and genotypic approach might help to identify *A. phocisimile* in future.

1. Introduction

Genus *Arcanobacterium* comprises the species *Arcanobacterium haemolyticum, Arcanobacterium hippocoleae, Arcanobacterium pluranimalium,* and *Arcanobacterium phocae* [1]. More recently *A. canis* and *A. phocisimile* were described as novel species of this genus [2, 3]. *Arcanobacterium pyogenes* together with *Arcanobacterium bernardiae, Arcanobacterium bonasi,* and *Arcanobacterium bialowiezense* was reclassified to the newly described species *Trueperella* [1]. The original description of *A. phocisimile* was based on physiological and biochemical characteristics, chemotaxonomic analysis, and 16S rDNA sequencing results of two strains isolated with several other bacterial species from a vaginal swab and an anal swab of two free living harbour seals of the German North Sea [3].

In the present study both initially described *A. phocisimile* strains and three additional strains obtained from three harbour seals were identified and further characterized phenotypically by MALDI-TOF MS analysis and genotypically by amplification and sequencing of various molecular targets.

2. Materials and Methods

2.1. Bacterial Strains. The *A. phocisimile* strains used in the present study included the previously described type strains *A. phocisimile* 2698^T (LMG 27073^T; CCM 8430^T) and *A. phocisimile* 4112 [3]. Additionally investigated *A. phocisimile* 3047 was isolated (post mortem) together with *Bacillus* spp., *Enterococcus* spp., *Erysipelothrix rhusiopathiae,* and α-haemolytic streptococci in the year 2005 from the lung of a female harbour seal with bronchopneumonia and perforation

Table 1: Oligonucleotide primer sequences and PCR conditions of the target genes used in the present study.

Oligonucleotide primers	Sequence	Program*	Expected size of PCR product (bp)	References
(1) 16S rDNA UNI-L (2) 16S rDNA UNI-R (amplification primer)	5′-AGAGTTTGATCATGGCTCAG-3′ 5′-GTGTGACGGGCGGTGTGTAC-3′	1	1,403	[4]
(3) 16S rDNA-533F (4) 16S rDNA-907R (sequencing primer)	5′-GTGCCAGCMGCCGCGGTAA-3′ 5′-CCGTCAATTCMTTTGAGTTT-3′	—	—	[4]
(5) Gap-F (6) Gap-R (*gap* primer)	5′-TCGAAGTTGTTGCAGTTAACGA-3′ 5′-CCATTCGTTGTCGTACCAAG-3′	2	830	[4]
(7) ISR-23S-F (8) ISR-23S-R (intergenic spacer region primer)	5′-CCTAGCCTGGTGGTTGGGTAG-3′ 5′-GTGCGGGTAACCAGAAATAACTCTG-3′	3	345	[6]
(9) C2700F (10) C3130R (*rpoB* primer)	5′-CGWATGAACATYGGBCAGGT-3′ 5′-TCCATYTCRCCRAARCGCTG-3′	4	406	[7]

*PCR program 1: x1 (95°C, 600 sec), x30 (95°C, 30 sec, 58°C, 60 sec, 72°C, 60 sec), and x1 (72°C, 420 sec). 2: x1 (94°C, 180 sec), x30 (94°C, 30 sec, 50°C, 40 sec, 72°C, 60 sec), and x1 (72°C, 300 sec). 3: x1 (95°C, 240 sec), x30 (95°C, 8 sec, 66°C, 10 sec, 72°C, 10 sec), x1 (72°C, 420 sec). 4: x1 (95°C, 600 sec), x35 (94°C, 30 sec, 50°C, 30 sec, 72°C, 120 sec), and x1 (72°C, 600 sec).

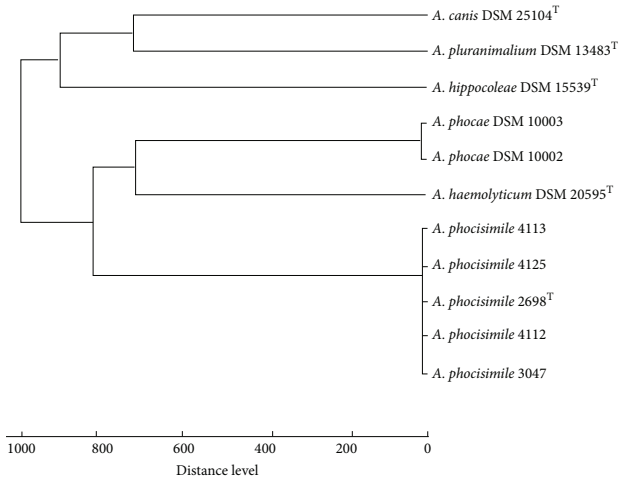

Figure 1: MALDI-TOF MS spectra from *A. phocisimile* 4113, *A. phocisimile* 4125, type strain *A. phocisimile* 2698^T, *A. phocisimile* 4112, *A. phocisimile* 3047, and all other species of genus *Arcanobacterium*.

of stomach. The harbour seal was found dead in Rantum, Sylt of the German North Sea. Also investigated *A. phocisimile* 4113 was recovered together with *Pasteurella* spp. and α-haemolytic streptococci from an anal swab and *A. phocisimile* 4125 together with *Pseudomonas* spp. and α-haemolytic streptococci also from an anal swab of two apparently healthy female harbour seals, respectively. *A. phocisimile* 4113 and *A. phocisimile* 4125 were isolated in 2007 during a monitoring program of free living harbour seals of the German North Sea.

2.2. Phenotypic and Genotypic Identification. All three newly investigated *A. phocisimile* strains were initially characterized phenotypically and by 16S rDNA sequencing [3, 4]. Both *A. phocisimile* strains previously mentioned in the species description [3] and the three *A. phocisimile* strains of the present study were further analysed by MALDI-TOF MS [5] and genotypically by amplification and sequencing of the previously described molecular target 16S-23S rDNA intergenic spacer region (ISR) and the genes *rpoB* and *gap* [4, 6, 7].

Table 2: Phenotypical properties of three *A. phocisimile* strains investigated in the present study and *A. phocisimile* 4112 and *A. phocisimile* 2698^T described previously.

Phenotypic properties	3047	4113	4125	4112**	2698^T**
Hemolysis on sheep blood agar	+	+	+	+	+
Hemolysis on rabbit blood agar	+	+	+	+	+
CAMP-like reaction with:*					
Staphylococcus aureus β-hemolysin	−	−	−	−	−
Streptococcus agalactiae	+	+	+	+	+
Rhodococcus equi	+	+	+	+	+
Arcanobacterium haemolyticum	−	−	−	−	−
Reverse CAMP reaction	+	+	+	+	+
Nitrate reduction	−[1]	−[1]	−[1]	−[1]	−[1]
Pyrazinamidase	(+)[1]	(+)[1]	+[1]	+[1]	+[1]
Pyrrolidonyl arylamidase	−[1]	−[1]	(+)[1]	−[1]	−[1]
Alkaline phosphatase	−[1]	−[1]	−[1]	(+)[1]	(+)[1]
β-Glucuronidase (β-GUR)	−[1,3]	−[1,3]	−[1,3]	−[1,3]	−[1,3]
β-Galactosidase (β-GAL)	+[1,3]	+[1,3]	+[1,3]	+[1,3]	+[1,3]
α-Glucosidase (α-GLU)	+[1,2,3]	+[1,2,3]	+[1,2,3]	+[1,2,3]	+[1,2,3]
β-Glucosidase (β-GLU)	−[2]	−[2]	−[2]	−[2]	−[2]
N-Acetyl-β-glucosaminidase (β-NAG)	−[1], +[3]	−[1], +[3]	−[1], +[3]	−[1], +[3]	−[1], +[3]
Esculin (β-glucosidase)	−[1]	−[1]	−[1]	−[1]	−[1]
Urease	−[1]	−[1]	+[1]	−[1]	−[1]
Gelatine	−[1]	−[1]	−[1]	−[1]	−[1]
Fermentation of:					
D-Glucose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Ribose	−[1]	+[1]	+[1]	+[1]	+[1]
D-Xylose	−[1]	−[1]	−[1]	−[1]	−[1]
D-Mannitol	−[1]	−[1]	−[1]	−[1]	−[1]
D-Maltose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Lactose	+[1]	+[1]	+[1]	+[1]	+[1]
D-Saccharose	+[1]	+[1]	+[1]	+[1]	+[1]
Glycogen	+[1]	+[1]	+[1]	+[1]	+[1]
α-Mannosidase	+[2]	+[2]	+[2]	+[2]	+[2]
Catalase	+	+	+	+	+
Serolysis on Loeffler agar	−	−	−	−	−
Caseinase	−	−	−	−	−
Starch hydrolysis	+	+	+	+	+

The reactions are shown as follows: *synergistic CAMP-like reaction with indicator strains; **results mostly obtained from Hijazin et al., 2013 [3]; +: positive reaction; (+): weak positive reaction; −: negative reaction; 1: Api Coryne test system (Biomerieux, Nürtingen, Germany); 2: tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); 3: 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

The primer sequences and the thermocycler programs are given in Table 1.

3. Results and Discussion

All three strains newly characterized in the present study could reliably be identified as *A. phocisimile* by phenotypic properties and by 16S rDNA sequencing. The phenotypic properties appeared to be almost identical to both previously characterized *A. phocisimile* strains (Table 2). However, a positive pyrazinamidase reaction of *A. phocisimile* seems to be the only reliable biochemical property for differentiation of *A. phocisimile* from pyrazinamidase negative *A. phocae*.

As shown by numerous authors MALDI-TOF MS is a powerful tool for species characterization of a broad spectrum of gram-positive and gram-negative bacteria [8–10]. This technique had previously been successfully used for rapid and reliable identification of bacteria of genera *Arcanobacterium* and *Trueperella* [5, 11]. The MALDI-TOF MS analysis of the present study revealed that by using the current Bruker data base, all five strains of this hitherto unknown species could not be identified to species level. However, using the MALDI Biotyper 3.1 software package the log (score) values of *A. phocisimile* 4112, *A. phocisimile* 3047, *A. phocisimile* 4113, and *A. phocisimile* 4125 matched against *A. phocisimile* 2698^T with log (score) values between 2.69

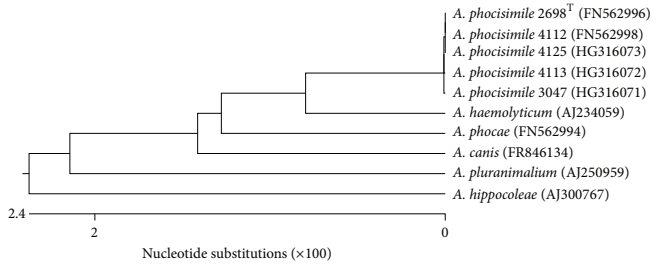

Figure 2: Dendrogram analysis of 16S rDNA sequences of the *A. phocisimile* strains of the present study and reference strains of genus *Arcanobacterium* obtained from NCBI GenBank.

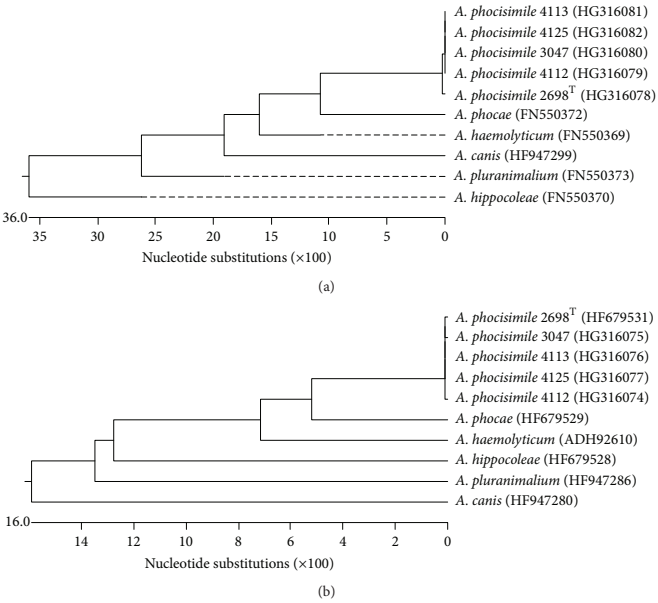

Figure 3: Dendrogram analysis of sequences of the genes *rpoB* (a) and *gap* (b) of the *A. phocisimile* strains of the present study and all other species of genus *Arcanobacterium* obtained from NCBI GenBank.

and 2.74 indicating that all five strains belong to this newly described species. Inclusion of *A. phocisimile* in the Bruker reference database will allow for the identification of this new species in future. A dendrogram analysis of the MALDI-TOF MS results is presented in Figure 1.

The genotypic classification by 16S rDNA sequencing revealed that the three novel *A. phocisimile* strains of the present study yielded 100% identity to both *A. phocisimile* strains described previously [3], also including the type strain *A. phocisimile* 2698^T (Figure 2).

Comparable to previously described *A. canis* [11] all five *A. phocisimile* from the present study could additionally be classified by amplification and sequencing of ISR (FN563000, FN563002, HG316083, HG316084, and HG316085), gene *rpoB* (HG316078, HG316079, HG316080, HG316081, and HG316082), and gene *gap* (HF679531, HG316074, HG316075, HG316076, and HG316077) yielding for all three molecular targets an identity of ≥99.4%, ≥99.8%, and ≥99.8%, respectively, for all five strains among each other. A typical dendrogram using the sequencing results of the target genes *rpoB* and *gap* is shown in Figure 3.

The results of the present study revealed that phenotypic properties, the determination of peptidic spectra by MALDI-TOF MS, and the various genotypic targets allow for a reliable identification of *A. phocisimile* and a further differentiation of *A. phocisimile* from closely related *A. phocae* which could also be isolated from marine mammals [12]. However, all *A. phocisimile* strains of the present study were isolated together with various other bacteria, partly from obviously healthy animals, indicating that the pathogenic importance of this species for marine mammals remains unclear.

4. Interpretive Summary

Arcanobacterium phocisimile type strain and four additional *A. phocisimile* strains isolated from harbour seals were identified phenotypically, by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), by sequencing 16S rDNA, and, as novel molecular targets, by sequencing 16S-23S rDNA intergenic spacer region and the genes *rpoB* and *gap* indicating that MALDI TOF MS and the molecular targets might help to identify this novel species.

Conflict of Interests

The authors declare that they have no conflict of interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the paper.

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Arcanobacterium pinnipediorum sp. nov., isolated from a harbour seal

Osama Sammra[1], Anna Balbutskaya [1], Hivda Ülbegi-Mohyla[1], Samy Nagib[1], Christoph Lämmler[1*], Peter Kämpfer[2], Stefanie P. Glaeser[2], Jan Golke[3], Hans-Jürgen Busse[3], Ellen Prenger-Berninghoff[4], Amir Abdulmawjood[5]

[1] Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen,35392 Gießen, Germany

[2] Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany

[3]Abteilung für Klinische Mikrobiologie und Infektionsbiologie, Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität Wien, A-1210 Wien, Austria

[4] Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, 35392 Gießen, Germany

[5] Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, 30173 Hannover, Germany

* Corresponding author. Tel.: +49 6419938406; fax: +49 6419938409.
E-mail address: Christoph.Laemmler@vetmed.uni-giessen.de

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2710^T is KJ596349.

One supplementary table is available with the online Supplementary Material.

Abstract

A polyphasic taxonomic study was performed on an unidentified *Arcanobacterium*-like Gram-stain positive bacterium, assigned strain 2710^T, isolated from a harbor seal. Comparative 16S rRNA gene sequence analysis showed that this bacterial strain belonged to the genus *Arcanobacterium* most closely related to the type strains of *Arcanobacterium phocae* (98.4% 16S rRNA gene sequence identity) and *Arcanobacterium phocisimile* (97.5%). Sequence similarities to type strains of other *Arcanobacterium* species were between 95.3 and 96.9%. Hybridization values for strain 2710^T and *A. phocae* DSM 10002^T, and for strain 2710^T and *A. phocisimile* LMG 27073^T were 4.7% (reciprocal 56%) and 23% (reciprocal 7.7%), respectively.

The presence of the major menaquinone MK-9(H$_4$) and a polar lipid profile with the major compounds diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol-mannoside supported the affiliation of the isolate 2710^T with the genus *Arcanobacterium*.

The major fatty acids were C$_{16:0}$, C$_{18:1}\omega$9c, C$_{18:0}$, and C$_{18:2}\omega$6,9c/ anteiso-C$_{18:0}$.

The peptidoglycan structure was of cross-linkage type A5α L-Lys-L-Lys-D-Glu.

Physiological and biochemical tests clearly distinguished the isolate from other members of the genus *Arcanobacterium*. Based on these tests, it is proposed that this unknown bacterium should be classified as a novel species with the name *Arcanobacterium pinnipediorum* sp. nov. The type strain of *Arcanobacterium pinnipediorum* is 2710^T (=DSM 28752^T, =LMG 28298^T).

Introduction

The genus *Arcanobacterium* was described by Collins *et al*. (1982) to accommodate a group of facultatively anaerobic, asporogenous Gram-stain-positive rods. Yassin *et al.* (2011) proposed twas in need of a taxonomic revision. These authors proposed, also supported by different quinone systems, that genus *Arcanobacterium* should be split into two genera, with *A. haemolyticum, A. phocae, A. pluranimalium* and *A. hippocoleae* belonging to genuat the genus was in need of taxonomic revisin, supported by different quinone systems, and should be split into two genera, with *Arcanobacterium haemolyticum*, *A. phocae*, *A. pluranimaliu*m and *A. hippocoleae* belong to the genus *Arcanobacterium* sensu stricto and *A. abortisuis*, *A. bernardiae, A. bialowiezense, A. bonasi* and *A. pyogenes* being transferred to genus *Trueperella*. More recently, *Arcanobacterium canis* and *Arcanobacterium phocisimile* were described as novel species of genus *Arcanobacterium* (Hijazin *et al*., 2012, 2013).

During routine microbiological diagnostic studies, one bacterial strain isolated from a harbour seal showed unusual features. As previously described for *A. phocisimile* (Hijazin *et al*., 2013), this strain was isolated, together with several others (data not shown), during a monitoring programme of free-living harbour seals of the German North Sea. Strain 2710^T was isolated in 2004 from an anal swab of a living male harbour seal (length 180 cm, weigh 77 kg). The strain was characterized based on Gram-staining and phenotypically based on haemolytic properties, CAMP-like haemolytic reactions and determination of biochemical properties using the API Coryne system (bioMerieux) and various other analytical procedures (Ülbegi-Mohyla *et al*., 2009; Hijazin *et al*., 2013). The strain was additionally characterized genotypically based on 16S rRNA gene sequencing (Sammra *et al*., 2014).

The 16S rRNA gene fragment sequenced for strain 2710^T represented a continuous stretch of 1348 bp (gene sequence positions 28-1392, according to the *Escherichia coli* numbering given by Brosius *et al*., 1978). Detailed phylogenetic analysis was performed with the ARB software package release 5.2 (Ludwig *et al*., 2004) using the database of the “All-Species Living Tree" Project (LTP; Yarza *et al*., 2008) release LTPs111 (February 2013). The sequence of the new strain was aligned with SINA (v1.2.11) according to the SILVA seed alignment (http://www.arb-silva.de; Pruesse *et al*., 2012) and implemented into the LTP database. The alignment of all sequences included in the phylogenetic analysis was checked manually based on secondary structure information. A maximum-parsimony tree was reconstructed using DNAPARS version 3.6 (Felsenstein, 2005), a maximum-likelihood tree using RAxML v7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, and a neighbour-joining (generated in ARB neighbour-joining) using the Jukes-Cantor correction as the evolutionary model (Jukes & Cantor,

1969) with 100 resamplings (Bootstrap analysis; Felsenstein, 1985). All calculations were based on 16S rRNA gene sequences between gene positions 132 to 1379 (according to *E. coli* numbering, Brosius *et al*., 1978). Phylogenetic trees showed that strain 2710^T was placed into the monophyletic cluster of the genus *Arcanobacterium* clustering with high bootstrap support together with the type strain of *A. phocae* (Fig. 1). Pairwise sequence similarity analysis (calculated in the ARB neighbor-joining tool without the use of an evolutionary model) showed that strain 2710^T shared highest 16S rRNA gene sequence similarity with the type strains of *A. phocae* and *A. phocisimile* (98.4 and 97.5%, respectively). Sequence similarities to all other type strains of *Arcanobacterium* species ranged from 95.3 and 96.9%. Strain 2710^T shared all 16S rRNA gene sequence signature nucleotides defined as specific for the genus *Arcanobacterium* (Subcluster I; Yassin *et al*., 2011), which distinguish the genera *Arcanobacterium* and *Trueperella* (Yassin *et al.*, 2011) (Table S1, available in the online Supplementary Material).

For further genotypic differentiation between strain 2710^T and those type strains sharing more than 97% 16S rRNA gene sequence similarity, genomic DNA was extracted according to Pitcher *et al.* (1989) and DNA DNA hybridization (DDH) was performed as described by Ziemke *et al.* (1998). Hybridization values between strain 2710^T and *A. phocae* DSM 10002^T and *A. phocisimile* LMG 27073^T were 4.7% (reciprocal 56%) and 23% (reciprocal 7.7%), respectively.

Quinones and polar lipids were extracted by an integrated procedure and analysed as previously described (Tindall, 1990a, b; Altenburger *et al*., 1996). For HPLC analysis of the quinone system, the equipment applied was as described by Stolz *et al*. (2007). The quinone system contained the major compound menaquinone MK-9(H_4) (91%) with minor amounts of MK-9 (6%) and MK-8(H_4) (3%). In the polar lipid profile diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside were predominant. In addition, moderate to minor amounts of five unidentified glycolipids, three unidentified aminolipids and five unidentified polar lipids, only detectable after total lipid staining, were detected (Fig. 2).

For fatty acid analysis, biomass of strain 2710^T was cultured on sheep blood agar at 37 °C for 72 h and harvested in approximately the same growth stage as reported for other ther *Arcanobacterium* species. Extraction and analysis of fatty acids was performed as described previously (Kämpfer & Kroppenstedt, 1996) using an HP 6890 gas chromatograph with a Sherlock MIDI software version 2.11 and a TSBA peak naming table version 4.1. The fatty acid profile of strain 2710^T contained $C_{16:0}$ (32.5%), $C_{18:1}\omega9c$ (24.9%), $C_{18:0}$ (22.7%) and $C_{18:2}\omega6,9c$/anteiso-$C_{18:0}$ (summed feature 5; 12.9%) as major components and $C_{14:0}$, $C_{12:0}$ and $C_{10:0}$ (all

below 4%) as minor components, and was in congruence with those reported for other *Arcanobacterium* species (Yassin *et al.,* 2011 Hijazin *et al.,* 2012; 2013).

Peptidoglycan analysis was performed according to Schumann (2011). The peptidoglycan structure was of corss-linkage type A5α ($_{L}$-Lys-$_{L}$-Lys-$_{D}$-Glu) (A11.51 according to www.peptidoglycan-types.info) and matched the description of the genus *Arcanobacteriu*m as emended by Yassin *et al.* (2011). Physiological characterization were performed as described previously (Hijazin *et al.,* 2012; 2013), results for strain 2710^{T} are given in the species description and are compared with the type strains of other *Arcanobacterium* species in Table 1.

Based on these the genotypic, chemotaxonomic and physiological data, strain 2710^{T} is considered to represent a novel species of the genus *Arcanobacterium* for which the name Arcanobacterium pinnipediorum sp. nov. is proposed.

Description of *Arcanobacterium pinnipediorum* sp. nov.

Arcanobacterium pinnipediorum [(pin.ni.pe.di.o'rum. N.L. gen. pl. n. pinnipediorum, of Pinnipedia, a group of semi-aquatic marine animals].

Cells are non-motile, non-spore-forming, Gram-stain-positive, pleomorphic, v-shaped, oxidase-negative rods. On sheep and rabbit blood agar, forms discoid, opaque and whitish pinpoint colonies, 0.5 mm in diameter, with a weak zone of hemolysis. Growth occurs under microaerobic conditions; less pronounced growth under aerobic and anaerobic conditions. CAMP-like reactions occurr with *Streptococcus agalactiae* and *Rhodococcus equi* as indicator strains and a reverse CAMP reaction in a zone of staphylococcal *β*-haemolysin is observed. Acid is produced or weakly produced from D-glucose, maltose, D-lactose, D-xylose, sucrose and glycogen, but not from D-ribose or D-mannitol. Activity of the following enzymes is observed: pyrrolidonyl arylamidase, pyrazinamidase, alkaline phosphatase, *β*-galactosidase, *α*-glucosidase, *N*-acetyl-*β*-glucosaminidase, catalase and amylase. No activity was detected for the following enzymes: nitrate reductase, *β*-glucuronidase, *β*-glucosidase and *α*-mannosidase. Negative for aesculin hydrolysis, urease, gelatinase, serolysis on Loeffler agar and caseinase. The quinone system contains predominantly MK-9(H_4) and minor amounts of MK-9 and MK-8(H_4). In the polar lipid profile diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside are the major components. Moderate to minor amounts of five unidentified

glycolipids, three unidentified aminolipids and five unidentified polar lipids, only detectable after total lipid staining, are detectable.

Major fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$, $C_{18:0}$, and summed in feature 5 ($C_{18:2}\omega6,9c$/anteiso-$C_{18:0}$); minor fatty acids are $C_{14:0}$, $C_{12:0}$ and $C_{10:0}$.

The peptidoglycan structure is of cross-linkage type A5α (L-Lys-L-Lys-D-Glu) (A11.51 according to www.peptidoglycan-types.info).

The type strain, 2710^T (=DSM 28752, =LMG 28298), was isolated from an anal swab of a harbour seal (*Phoca vitulina*). The 16S rRNA gene sequence of the type strain contains all 12 16S rRNA gene signature nucleotides defined by Yassin *et al.* (2011) for the genus *Arcanobacterium* (Subcluster I, Yassin *et al*., 2011) that distinguish species of the genus *Arcanobacterium* from species of the genus *Trueperella*.

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Table 1 Phenotypical properties of strain 2710^T compared with the type strains of other species of the genus *Arcanobacterium*.

Strain: 1, 2710^T; 2, *A. phocae* DSM 10002^T; 3, *A. phocisimile* DSM 26142^T; 4, *A. canis* DSM 25104^T; 5, *A. haemolyticum* DSM 20595^T; 6, *A. hippocoleae* DSM 15539^T; 7, *A. pluranimalium* DSM 13483^T. + +, enhanced positive reaction; + (+), slightly enhanced positive reaction; +, positive reaction; (+), weak positive reaction; -, negative reaction.

Phenotypic properties	1	2**	3**	4**	5**	6**	7**
Hemolysis on sheep blood agar	+	+(+)	+	+	+	(+)	+
Hemolysis on rabbit blood agar	+	+(+)	+	+(+)	++	+	+(+)
CAMP-like reaction with:**							
Staphylococcus aureus β-hemolysin	-	-	-	(+)	-	+	+
Streptococcus agalactiae	+	+	+	-	+	-	-
Rhodococcus equi	+	+	+	+	+	-	+
Arcanobacterium haemolyticum	-	-	-	-	-	+	+
Reverse CAMP reaction	+	+	+	-	+	-	-
Nitrate reduction	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
Pyrazinamidase	+[a]	-[a]	+[a]	-[a]	+[a]	-[a]	+[a]
Pyrrolidonyl arylamidase	+[a]	-[a], +[b]	-[a]	-[a]	-[a]	-[a,b]	+[a,b]
Alkaline phosphatase	+[a]	+[a,b]	(+)[a]	+[a]	+[a]	+[a,b]	-[a,b]
β-Glucuronidase	-[a,c]	-[a,c]	-[a,c]	+[a,b,c]	-[a,b,c]	+[a,c]	+[a,b,c]
β-Galactosidase	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	-[a], (+)[c]
α-Glucosidase	+[a,c]	+[a,c]	+[a,b,c]	+[a,c]	+[a,b,c]	+[a,c]	-[a,b,c]
β-Glucosidase	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	+[b]
N-acetyl-*β*-Glucosaminidase	+[a,c]	+[a,c]	-[a], +[c]	+[a,c]	+[a,c]	+[a,c]	-[a,c]
Hydrolysis of:							
Aesculin	-[a]	-[a]	-[a]	-[a]	-[a]	(+)[a]	+[a]
Urease	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
Gelatine	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	+[a]
Acid production from:							
D-Glucose	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
D-Ribose	-[a]	+[a]	+[a]	+[a]	+[a]	-[a]	+[a]
D-Xylose	(+)[a]	(+)[a]	-[a]	-[a]	-[a]	-[a]	-[a]
D-Mannitol	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
Maltose	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	(+)[a]
Lactose	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	-[a]
Sucrose	(+)[a]	+[a]	+[a]	+[a]	-[a]	-[a]	-[a]
Glycogen	+[a]	+[a]	+[a]	+[a]	-[a]	-[a]	-[a]
α-Mannosidase	-[b]	+[b]	+[b]	+[b]	+[b]	-[b]	+[b]
Catalase	+	+	+	-	-	-	+
Serolysis on Loeffler agar	-	-	-	-	-	-	-
Caseinase	-	-	-	-	-	-	+
Amylase	(+)	+	+	+	-	+	+

*Synergistic CAMP-like reaction with indicator strains

†Results obtained with: a, API Coryne test system (bioMerieux); b, tablets containing substrates (Rosco Diagnostica); c, 4-methylumbelliferyl-conjugated substrates (Sigma).

**Data for the reference strains are taken from Ulbegi-Mohyla *et al.* (2009) and Hijazin *et al.* (2013)

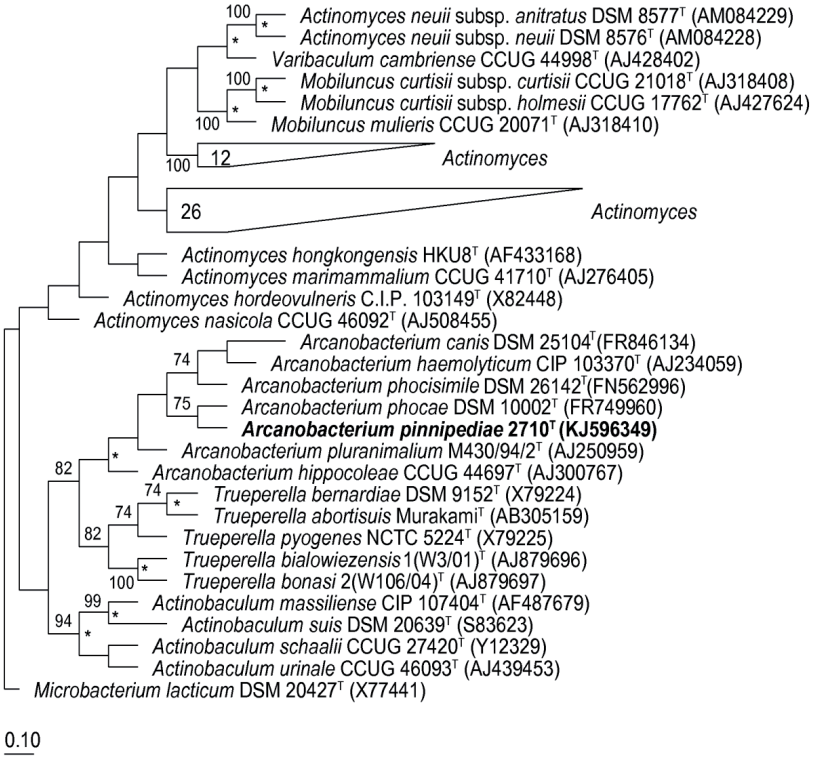

Fig. 1. Maximum-parsimony tree based on nearly full length 16S rRNA gene sequences showing the phylogenetic position of strain 2710^T within the family *Actinomycetaceae*. The tree was generated in ARB and based on *E. coli* sequence positions 128-1379 (Brosius *et al*., 1978). Numbers given at nodes represent bootstrap percentage (>70%) calculated based on 100 replications. Asterisk indicates nodes that also showed a high bootstrap support in the maximum-likelihood tree generated with RAxML. The 16S rRNA gene sequence of *Microbacterium lacticum* DSM 20427^T was used as outgroup. Bar, 0.1 substitutions per nucleotide sequence position.

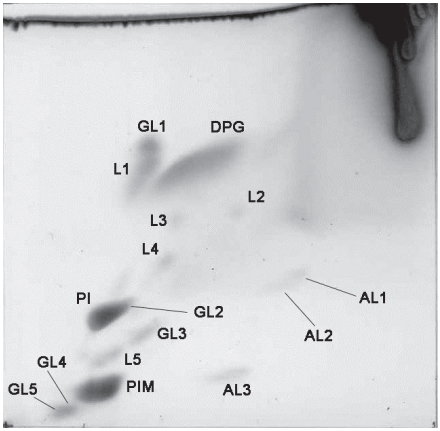

Fig. 2: Total polar lipid profile of strain 2710^T after two-dimensional TLC and staining with molybdatophosphoric acid. DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol-mannoside; AL1-3, unidentified aminolipids; GL1-5, unidentified glycolipids; L1-5, unidentified polar lipids detectable only after total lipid staining.

Supplementary Table 1: Assignment of 16S rRNA gene signature nucleotides for strain 2710^T The twelve listed signature nucleotides were determined by Yassin *et al*. (2011) to distinguish species of the genera *Arcanobacterium* and *Trueperella*. 1: strain 2710^T, 2: *Arcanobacterium* (Subcluster I, Yassin *et al*., 2011), 3: *A. hippocoleae* CCUG 44697^T, 4: *A. canis* DSM 25104^T, 5: genus *Trueperella* (Subcluster II, Yassin *et al*., 2011), *Arcanobacterium hippocoleae* CCUG 44697^T and *A. canis* DSM 25104^T showed signature nucleotides of both genera (as described by Yassin *et al*., 2011, Hijazin *et al*., 2012). For positions 157-164, 440, and 598 signature nucleotides given by Yassin *et al*. (2011) for the genera *Arcanobacterium* and *Trueperella* were corrected according the nucleotide sequences of reference strains obtained in the ARB generated alignment as described previously (Hijazin *et al.,* 2012).

Nucleotide position*	1	2	3	4	5
157-164	U-G	U-G	U-G	G-U	G-U
440	U	U	C	U	C
443-491	C-G	C-G	U-G	C-G	U-G
446-488	U-G	U-G	A-U	U-G	A-U
492	U	U	C	U	C
497	U	U	G	U	G
598	U	*U*	U	U	*C*
625	C	C	C	C	U
631	C	C	C	C	U
659	C	C	C	C	U
776	G	G	G	G	U
1278	G	G	G	G	U

*Nucleotide position according to *E. coli* (Brosius *et al*., 1978) based on the alignment performed in ARB.

Arcanobacterium wilhelmae sp. nov., isolated from the genital tract of a rhinoceros (*Rhinoceros unicornis*)

Osama Sammra[1], Jörg Rau[2], Jörn-Peter Wickhorst[1], Mazen Alssahen[1], Abdulwahed Ahmed Hassan[1,3], , Christoph Lämmler[1*], Peter Kämpfer[4], Stefanie P. Glaeser[4], Hans-Jürgen Busse[5], Tanita Kleinhagauer[5], Tobias Knauf-Witzens[6], Ellen Prenger-Berninghoff[7] and Amir Abdulmawjood[8], Günter Klein[8]

[1] *Institut für Pharmakologie und Toxikologie, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany*

[2] *Chemisches und Veterinärunterschungsamt Stuttgart (CVUAS), Fellbach, Germany*

[3] *Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq*

[4] *Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany*

[5] *Institut für Mikrobiologie, Veterinärmedizinische Universität Wien, A-1210 Wien, Austria*

[6] *Wilhelma, Zoologisch-Botanischer Garten, Stuttgart, 70342 Stuttgart, Germany*

[7] *Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany*

[8] *Institut für Lebensmittelqualität und -sicherheit, Stiftung Tierärztliche Hochschule Hannover, 30173 Hannover, Germany*

*** Correspondence:** Christoph Lämmler, christoph.laemmler@vetmed.uni-giessen.de

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 647^T is LT160960.

Abstract

A taxonomic study using a polyphasic approach was performed on an unidentified *Arcanobacterium*-like Gram-stain-positive bacterium isolated from the genital tract of a rhinoceros. Comparative 16S rRNA gene sequencing showed that the bacterium belonged to the genus *Arcanobacterium* and was most closely related to the type strains of *Arcanobacterium canis* (98.8 % 16S rRNA gene sequence similarity), *Arcanobacterium phocisimile* (97.8 %), *Arcanobacterium phocae* (97.7 %), *Arcanobacterium haemolyticum* (97.4 %), *Arcanobacterium hippocoleae* (96.6 %), *Arcanobacterium pinnipediorum* (96.4 %) and *A. pluranimalium* (95.4 %). DNA-DNA hybridization values among strain 647^T and *Arcanobacterium canis* DSM 25104 were very low, 13.4% (reciprocal: 15.9%). The genomic DNA G+C content of strain 647^T was 58.7 mol%. The presence of the major menaquinone MK-9(H_4) supported the affiliation of this strain to the genus *Arcanobacterium*. The polar lipid profile consisted of the major components diphosphatidylglycerol, phosphatidylcholine and an unidentified phosphoglycolipid. The results of physiological and biochemical testing clearly distinguished that the unknown bacterium from other species of the genus *Arcanobacterium*. Based on these tests, it is proposed that the unknown bacterium should be classified as a representative of a novel species of the genus *Arcanbacterium wilhelmae* sp. nov. The type strain is 647^T (= DSM 102162^T =BCCM/LMG 29418^T).

Introduction

According to Yassin *et al.* [1] the genus *Arcanobacterium*, which was originally described by Collins *et al.* [2] was in need of a taxonomic revision. These authors split genus *Arcanobacterium* into two genera with *Arcanobacterium abortisuis, Arcanobacterium bernardiae, Arcanobacterium bialowiezense, Arcanobacterium bonasi* and *Arcanobacterium pyogenes* being transferred to a new genus, *Trueperella* and *Arcanobacterium haemolyticum, Arcanobacterium phocae, Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae* constituting the genus *Arcanobacterium* [1], with *A. haemolyticum* as the type strain for the genus. More recently, the novel species *Arcanobacterium canis, Arcanobacterium phocisimile and Arcanobacterium pinnipediorum* were described [3-5].

During a routine microbiological diagnostic test, a bacterial strain isolated from vaginal discharge of a rhinoceros, showed unusual features. Applying a polyphasic taxonomic approach, this strain could be classified as a representative of a novel species within the genus *Arcanobacterium*.

Strain 647^T was isolated in January 2012 in high numbers (+++) together with streptococci of serological group L (+++), *Streptococcus dysgalactiae* subsp. *equisimilis* (++) and *Escherichia coli* (++) from a vaginal swab of Asian rhinoceros (*Rhinoceros unicornis*) Sani from Stuttgart Zoo Wilhelma, Germany. Sani, after five regular calving with the last birth in 2008, did no longer display symptoms of heat. In addition, a yellow-beige muco-serous vaginal discharge was observed from October 2010 onwards. According to 16S rDNA gene sequencing results, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Fourier Transform Infrared (FTIR) Spectroscopy, the same isolate could also be recovered from various genital swabs of rhinoceros Sani on March 2013 (data not shown). Strain 647^T isolated in 2012 was used in the detailed characterization of the present study. The strain was cultured on sheep blood agar at 37 ºC for 48 h under microaerobic conditions in a candle jar. The strain was characterized phenotypically by Gram-staining, oxidation reaction [6], motility test [7], haemolysis, CAMP-like hemolytic properties and biochemical properties, also with the help of the API Coryne test system (bioMerieux) and by 16S rRNA gene sequencing [9,10].

The sequenced 16S rRNA gene sequence of strain 647^T was a continuous stretch of 1495 nt. Pairwise sequence similarities to closest related type strains were determined using EzTaxon. (www.ezbiocloud.net; [11]). Phylogenetic trees based on nearly full-length 16S rRNA gene

sequences were reconstructed with ARB release 5.2 [10] using the "All species living tree project" (LTPs; [12]) database release LTPs119 (July, 23 2015). Sequences not included in the database were added after alignment using the SILVA Incremental Aligner (SINA; v1.2.11; [13]). The alignment was re-checked manually and gene sequences between *Escherichia coli* positions 128 to 1379 (numbering according to Brosius *et al*., [14]) were included in the analysis. Phylogenetic trees were calculated with the maximum-parsimony method using DNAPARS v 3.6 [15], the maximum-likelihood method using RAxML version 7.04 [16] with GTR-GAMMA and rapid bootstrap analysis, and the neighbor-joining methods using ARB Neighbor-joining and the Jukes-Cantor correction [17]. The phylogenetic trees based on 100 resamplings (bootstap analysis; [18]). The 16S rRNA gene sequence-based phylogenetic trees showed the allocation of strain 647^T in the genus *Arcanobacterium* distinct from the genus *Trueperella* (Fig. 1, Fig. 2) In all calculated trees strain 647^T clustered with *A. canis* DSM 25104^T which was always supported by high bootstrap values (Fig. 1). Strain 647^T and *A. canis* DSM 25104^T shared 98.8% 16S rRNA gene sequence similarity while the sequence similarity to other strains of type species of the genus *Arcanobacterium* was in the range of 95.4 % to 97.8 %: *A. phocisimile* (97.8 %), *A. phocae* (97.7 %), *A. haemolyticum* (97.4 %), *A. hippocoleae* (96.6 %), *A. pinnipediorum* (96.4 %), and *A. pluranimalium* (95.4 %). Sequence similarities to type strains of members of the genus *Trueperella* ranged between 94.2 % and 95.0 %. Except for the pairwise sequence similarity of strain 647^T to *A. canis* DSM 25104^T, pairwise sequence similarities to other type strains were below the 98.65 % level of 16S rRNA gene sequence similarity that corresponds to the currently accepted ANI threshold for species demarcation [19]. In consequence, the genomic relatedness of strain 647^T and *A. canis* DSM 25104^T was investigated by DNA-DNA hybridization analysis according to the method of Ziemke *et al.* [20] with DNA extracted by the methods of Pitcher *et al.* [21]. The DNA-DNA hybridization values were very low, 13.4% (reciprocal: 15.9%), clearly indicating that the two strains represent two different species.

The G+C content of the genomic DNA of strain 647^T and A. canis DSM 25104^T was determined by the DNA melting temperature method established by Gonzales and Saiz-Jimenez [22] as described previously [23] except 20 % (v/v) formamide was used in the reaction buffer to enable a better melting of the high G+C content genomic DNA. The obtained G+C content of strain 647^T was 85.7 mol% and that for *A. ca*nis DSM 25104^T was 51.1 mo%. The DNA G+C content of strain 647^T was slightly above the reported for species of the genus *Arcanobacterium*, while the G+C content determined for *A. canis* DSM 25104^T was in the reported range of 50-57 mol% [1]. The

large difference among the genomic G+C contents of strain 647^T and *A. canis* DSM 25104^T again supported the finding that the two strains represent separate species.

The results of physiological characterization are shown in Table 1 and also in the species description. In addition, the novel species displayed no CAMP-like hemolytic reactions or a reverse CAMP reaction which are well known to be used for phenotypical identification of bacteria of genus *Arcanobacterium* [8].

Quinones and polar lipids were extracted and analyzed applying an integrated method described by Tindall [24, 25] and Altenburger *et al*. [26]. HPLC analysis was carried out as described by Stolz *et al*. [27]. The quinone system of strain 647^T was composed of 71.3% MK-9(H_4), 17.3% MK-8(H_4), 8.4% MK-9, and 2.9% MK-9(H_2), which is in agreement with quinone systems of species of the genus *Arcanobacterium* and its emended genus description [1]. The polar lipid profile (Fig 2) contained the major lipids diphosphatidylglycerol (DPG), phosphatidylcholine (PC) and an unidentified phosphoglycolipid (PGL4). In addition, moderate amounts of an unidentified phospholipid (PL1), phosphatidylinositol and phosphatidylinositol-mannosides and minor amounts of three unidentified phosphoglycolipids (PGL1-3) and one glycolipid (GL1) were detected. The presence of the major compounds diphosphatidylglycerol and phosphatidylcholine as well as the presence of lesser amounts of phosphatidylinositol, a phosphatidylinositol-mannoside, PGL1 and GL1 and the absence of phosphatidylglycerol was also reported for *A. canis* [3]. These shared lipids might be regarded to reflect the close phylogenetic relatedness between strain 647^T and *A. canis*. However, the presence of the three phosphoglycolipids PGL2-4 in strain 647^T clearly distinguished the two strains.

Fatty acid analysis were performed as described previously [28]. Biomass of strain 647^T was cultured on sheep blood agar at 37 °C for 72 h and harvested at approximately the same growth stage as reported for all other species of the genus *Arcanobacterium*. Fatty acid profiling was performed in a H P 6890 gas chromatograph using the Sherlock MIDI software version 2.11 and a TSBA peak naming table version 4.1 for analysis. The fatty acid profile of strain 647^T consisted of $C_{16:0}$ (32.1 %), $C_{18:0}$ (21.8 %) and $C_{14:0}$ (19.5%), and $C_{18:1}$ ω9c (18.5 %), and $C_{18:2}$ ω 6,9 c/anteiso-$C_{18:0}$ (summed feature 5; 5.7 %) as major components, and $C_{12:0}$ (2.5 %) as minor components. This was congruent to fatty acid profiles of other *Arcanobacterium* species [1, 3, 5]. Fatty acid profiles published by Hijazin *et al*. [3] and Sammra *et al.* [5] were analyzed in the same laboratory as the data represented here by the use of biomass grown under the same conditions and harvested at the same growth stage.

Peptidoglycan analysis was fulfilled as stated by Schumann [29]. The peptidoglycan structure revealed a cross-linkage type A5α ($_{L}$-Lys-$_{L}$-Ala-$_{L}$-Lys-$_{D}$-Glu) (A11.53 according to www.peptidoglycan-types.info) and was correspondent to the description of the genus *Arcanobacteriu*m as rectified by Yassin *et al.* [1]. Physiological characterization were implemented as described heretofore [3,4]; results for strain 2710^{T} are given in the species description and are correlated with the type strains of other *Arcanobacterium* species in Table 1.

Based on these taxonomic studies, strain 647^{T} represents a novel species of genus *Arcanobacterium*, for which the name *Arcanobacterium wilhelmae* sp. nov. is proposed.

Description of *Arcanobacterium wilhelmae* sp. nov.

Arcanobacterium wilhelmae [(wil.'hel.mae. N.L. gen. n. *wilhelma*, of the Wilhelma zoo in Stuttgart from where the strain was isolated.

Cells are Gram-stain-positive, oxidase negative, non-motile, non-spore-forming rods (1-2 µm long and 0.5 µm wide). No CAMP-like reactions are observed with any of the indicator strains *Staphylococcus aureus*, *Streptococcus agalactiae* and *Rhodococcus equi*. Substrate utilization as sole carbon source is obtained from D-glucose, D-ribose, maltose, sucrose and glycogen, but not from D-xylose, D-mannitol and lactose. Activity of the following enzymes is observed: alkaline phosphatase, *β*-glucuronidase and *α*-glucosidase. No enzyme activity is detected for the following enzymes: nitrate reductase, pyrazinamidase, pyrrolidonyl arylamidase, *β*-galactosidase, *N*-Acetyl-*β*-glucoseaminidase, aesculin hydrolysis, for serolysis on loeffler agar, urease, gelatinase and catalase. Major fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ ω9c, and $C_{14:0}$ followed by summed feature 5 ($C_{18:2}\omega 6,9c$/anteiso-$C_{18:0}$); $C_{12:0}$ is present as minor fatty acid. The quinone system is composed of MK-9(H_4), MK-8(H_4), MK-9, and MK-9(H_2) (1:0.24:0.12:0.04). The polar lipid profile is composed of the major lipids diphosphatidylglycerol (DPG), phosphatidylcholine (PC) and the unidentified phosphoglycolipid (PGL4). In addition, moderate amounts of an unidentified phospholipid (PL1), phosphatidylinositol and phosphatidylinositol-mannosides and minor amounts of three unidentified phosphoglycolipids (PGL1-3) and one glycolipid (GL1) are detectable. The peptidoglycan structure is of cross-linkage type A5α ($_{L}$-Lys-$_{L}$-Ala-$_{L}$-Lys-$_{D}$-Glu) (A11.53 according to www.peptidoglycan-types.info).

The type strain, 647^{T} (= DSM 102162^{T} =BCCM/LMG 29418^{T}), was isolated from the genital tract of a female rhinoceros. The G+C content of the genomic DNA of the type strain is 58.7 mol%.

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Conflicts of interest

The authors declare that there is no conflicts of interest.

Ethical statement

The authors certify that the manuscript does not proclaim any work which requires approval by ethics committee(s).

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Table 1. Phenotypical properties of *Arcanobacterium wilhelmae* sp. nov., and various other species of genus *Arcanobacterium*.
Strains: 1, 647^T; 2, *A. canis* DSM 25104^T; 3, *A. phocae* DSM 10002^T; 4, *A. phocisimile* DSM 26142^T*; 5, *A. pinnipediorum* DSM 28752^T, 6, *A. haemolyticum* DSM 20595^T; 7, *A. hippocoleae* DSM 15539^T; 8, *A. pluranimalium* DSM 13483^T. Data for strains 2, 3, 4, 6, 7 and 8 are mostly obtained from Sammra *et al.* (2015). All strains are positive for utilization of D-glucose as sole carbon source (API Coryne), and negative for urease activity (API Coryne). ++, Enhanced positive, +(+), slightly enhanced positive; +, positive; (+), weakly positive; -, negative.

Phenotypic properties	1	2	3	4	5	6	7	8
Hemolysis on:								
Sheep blood agar	+	+	+(+)	+	+	+	(+)	+
Rabbit blood agar	+	+(+)	+(+)	+	+	++	+	+(+)
CAMP-like reaction with:*								
Staphylococcus aureus β-hemolysin	-	(+)	-	-	-	-	+	+
Streptococcus agalactiae	-	-	+	+	+	+	-	-
Rhodococcus equi	-	+	+	+	+	+	-	+
Arcanobacterium haemolyticum	-	-	-	-	-	-	+	+
Reverse CAMP reaction	-	-	+	+	-	+	-	-
Nitrate reduction	-†[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
Pyrazinamidase	-[a]	-[a]	-[a]	+[a]	+[a]	+[a]	-[a]	+[a]
Pyrrolidonyl arylamidase (API Coryne)	-[a]	-[a]	-[a]	-[a]	+[a]	-[a]	-[a]	+[a]
Alkaline phosphatase	+[a]	+[a]	+[a]	(+)[a]	+[a]	+[a]	+[a]	-[a]
β-Glucuronidase	+[a,b,c]	+[a,b,c]	-[a,c]	-[a,c]	-[a,c]	-[a,b,c]	+[a,c]	+[a,b,c]
β-Galactosidase	-[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	-[a], (+)[c]
α-Glucosidase	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	+[a,c]	-[a,b,c]
β-Glucosidase (substrate tables)	-	-	-	-	-	-	-	+
N-Acetyl-β-Glucosaminidase	-[a,c]	+[a,c]	+[a,c]	-[a], +[c]	+[a,c]	+[a,c]	+[a,c]	-[a,c]
Hydrolysis of aesculin (API Coryne)	-	-	-	-	-	-[1]	(+)	+
Hydrolysis of gelatin (API Coryne)	-	-	-	-	-	-	-	+
Substrate utilization as a sole carbon from (API Coryne):								
D-Ribose	+	+	+	+	-	+	-	+
D-Xylose	-	-	(+)	-	(+)	-	-	-
Maltose	+	+	+	+	+	+	+	(+)
Lactose	-	+	+	+	+	+	+	-
Sucrose	(+)	+	+	+	(+)	-	-	-
Glycogen	+	+	+	+	+	-	-	-
Catalase	-	-		+	+	-	-	+
Serolysis on Loeffler agar	-	-	-	-	-	-	-	-
Caseinase	-	-	-	-	-	-	-	+
Amylase	+	+	+	+	(+)	-	+	+

*Synergistic CAMP-like reaction with indicator strains

†Results determined with: a, API Coryne test system (bioMerieux); b, tablets containing substrates (Rosco Diagnostica A/S, Taastrup, Denmark); c, 4-methylumbelliferyl conjugated substrates (Sigma).

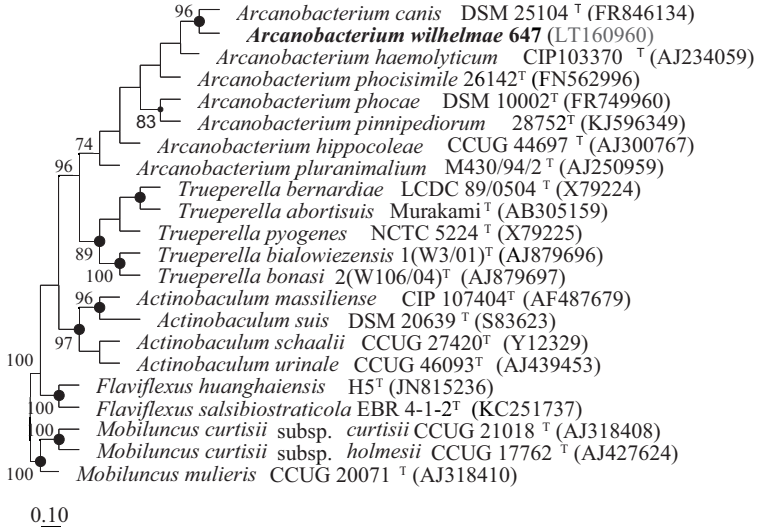


Fig. 1. Phylogenetic tree based on nearly full-length 16S rRNA gene sequences showing the position of strain 647^T within the genus *Arcanobacterium*. The tree was generated with the maximum-parsimony method using DNAPars in ARB. The tree based on DNA sequences among 16S rRNA gene termini 128 and 1379 (according to the *rrnB* gene sequence, Brosius *et al.*, 1978). Numbers at nodes represent bootstrap values based on 100 replications. Nodes marked with filled circles confirm phylogenetic trees generated with maximum-likelihood and neighbor-joining algorithms. Bar, 0.10 substitutions per nucleotide positions.

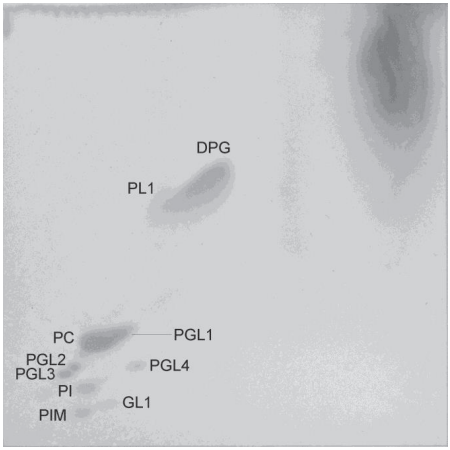


Fig. 2. Total polar lipid profile of strain 647^T after two dimensional thin-layer chromatography and staining with molybdatophosphoric acid. DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PIM, phosphatidylinositol-mannoside; GL, unidentified glycolipid; PI, phospholipids, PGL1-4, phosphoglycolipids.

Chapter 4 - Discussion

Taxonomic classification of genus *Arcanobacterium*

Genus Arcanobacterium (A.) comprises a group of facultatively anaerobic, Gram-positive rod-shaped bacteria that belong to the family *Actinomycetaceae*. The name of this genus originates from the word "*arcanus*", the Latin word for "secretive" or "mysterious". Genus *Arcanobacterium* was first described by Collins and coauthors (1982b) to include the novel species *Arcanobacterium haemolyticum*, a species precedently classified as *Corynebacterium haemolyticum* by MacLean et al. (1946) for strains isolated from infected wounds of American soldiers. Few years later, genus *Arcanobacterium* was expanded to include *A. pyogenes*, *A. bernardiae* and *A. phocae* isolated from infected wounds and abscesses of animals and from human origin, blood and abscesses from humans and multiple tissues of sea mammals, respectively, (Ramos et al., 1997) followed by *A. pluranimalium* from a porpoise and a deer (Lawson et al., 2001), *A. hippocoleae* from vaginal discharge of a mare (Hoyles et al., 2002), *A. bialowiezense* and *A. bonasi* from prepuce of European bison bulls (*Bison bonasus*) (Lehnen et al., 2006) and *A. abortisuis* from a placenta of a pig after abortion (Azuma et al., 2009). However, according to a proposal by Yassin and coauthors in 2011, based on chemotaxonomic and phylogenetic analysis, genus *Arcanobacterium* was reclassified to perpetuate *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocleae*, and transferring *A. pyogenes*, *A. bialowiezense*, *A. bonasi* and *A. abortisuis* to the forthwith described genus *Trueperella*, as *T. pyogenes*, *T. bialowiezensis*, *T. bonasi* and *T. abortisuis* (Yassin et al., 2011).

Phenotypic and genotypic characterization of *A. haemolyticum from humans and equines*

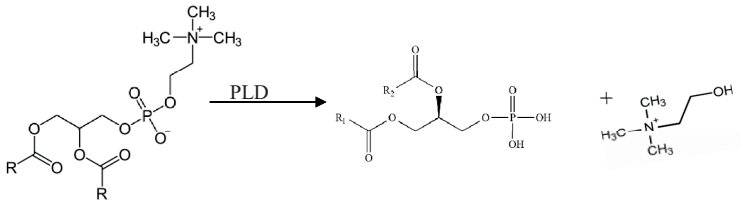
A. haemolyticum, was described as facultative anaerobic, Gram-positive, non-sporulating, non-motile, non-acid fast, catalase negative bacterium that was best cultured on sheep blood agar at an optimal temperature of 37 ºC after incubation for 48 h (Collins et al., 1982b; Lämmler and Hartwigk, 1995). *A. haemolyticum* exhibited enhanced hemolysis on rabbit blood agar compared to sheep blood agar (Lämmler and Blobel, 1988; Cummings et al., 1993; Ülbegi-Mohyla et al., 2009). In addition, *A. haemolyticum* interacted with staphylococcal β-hemolysin leading to a reverse CAMP reaction (Lämmler and Blobel, 1998; Lämmler, 1994; Ülbegi-Mohyla et al., 2009) which has been accredited to a reaction between phospholipase D (PLD) of *A. haemolyticum* and staphylococcal β-hemolysin (Souckova and Soucek, 1972).

Meanwhile, the submission of the complete genome of the type strain *A. haemolyticum* 11018^T (=DSM 20595^T) to GenBank (Yasawong et al., 2010), being the first member of its genus to be completely sequenced on the genome level, revealing more than 1,986,154 bp and 1,821 protein-coding genes, opened the way for further investigation of virulence and putative virulence factors of this species.

According to Jost and coauthors (2011), Arcanolysin (ALN), a latterly identified and well-characterized ~ 64 kDa in molecular mass cholesterol-dependent cytolysin (CDC) is a member of an exotoxin family causing pore formation to host cellular membrane. The CDCs also include intermedilysin (ILY) of *S. intermedius*, Listeriolysin O (LLO) of *L. monocytogenes*, perfringolysin O (PFO) of *C. perfringens*, pneumolysin (PLY) of *S. pneumoniae* and streptolysin O (SLO) of *S. pyogenes* and pyolysin (PLO) of *T. pyogenes* (Billington et al., 1997). ALN was believed to be prevalent in all *A. haemolyticum* strains and was highly correlated to PLO of *T. pyogenes*. The ALN encoding gene *aln*, measuring around 1,710 bp was flanked by two tRNA genes. It was neighbored from upstream by phosphoglycerate mutase gene (pgm; Arch_1063) and an alanine $tRNA_{GGC}$ and from downstream by a transcriptional terminator hypothetical protein with a signal sequence (Arch_1061), followed by an alanine $tRNA_{CGC}$. Both upper and downstream alanine tRNAs show a sequence identity of 91 % to each other. The 426 bp of Arch_1062 region, lying between Arch_1063 and Arch_1061, bears regulatory signals, a putative σ^{70} promoter and 30 direct repeats (ATTTT(G/C)(G/T/T) which are thought to play a transcription role in gene *aln* and resemble their counterparts present on the upstream region of PLO encoding gene *plo* of *T. pyogenes*. The % G + C value of 46.7 % for gene *aln* is considered relatively low in comparison to an average of 53.0 % for the whole genome. This low % G + C value of gene *aln* and existence of two flanking tRNA genes, which could operate as transgene insertion sites (Williams, 2002), raise a presumption that this gene may have been evolved in *A. haemolyticum* through horizontal gene transfer (Jost et al., 2011). An analysis of the primary structure of ALN protein revealed the presence of 569 amino acids, comprising 26 amino acid signal sequences that are predicted by SignalP. The ALN shared a 59.4 % and 71.5 % amino acid identity and similarity to its closest relative PLO of *T. pyogenes* and an average of ~ 50 % similarity to various other associates of the CDC family (Jost et al., 2011). The ALN protein also bears a putative PEST sequence and a variant undecapeptide within its domain 4, a well-known relevantly active toxin component. The ALN putative PEST sequence found within its N-terminus showed no similar existence on PLO of *T. pyogenes* but was rather found in LLO, an endotoxin secreted by *Listeria monocytogenes*. Referring to the pestfind algorithm, ALN displayed a score value of 7.58 compared to 4.71 for LLO of *L.*

monocytogenes, suggesting a high PEST sequence activity which could demonstrate the invasive role of *A. haemolyticum* to host cells (Lucas et al., 2010). The predicted ALN amino acid sequence revealed a variant undecapeptide within domain 4, resembling that of PLO, where both contained no conserved cysteine residue. Moreover, ALN was successfully cloned on *Escherichia coli* and was expressed as functional recombinant toxin, where it displayed an enhanced hemolytic and cellular activity on human, rabbit, porcine and equine blood and cell cultures compared to a less enhanced activity on sheep, cattle, mice and canine cells (Jost et al., 2011).

Phospholipase D (PLD), a 31.5 KDa pervasive protein, present in nearly all organisms, is a major phospholipid cleaving enzyme which plays a key role in hydrolyzing phosphatidylcholine (PA) into phosphatidic acid (PA) and choline. Phosphatidic acid generated within this common physiological process is crucial for the metabolism of lipids, cell signaling critical for restructuring of cytoskeleton, vesicular transition and incitation of cellular meiotic activity (Exton, 2002; Kolesnikov et al., 2012; Majd et al., 2013).



Phosphatidylcholine (PC) Phosphatidic acid Choline

Meanwhile, according to Souckova and Soucek (1972), PLD is considered the key factor of *A. haemolyticum* which reacts with staphylococcal β-hemolysin leading to a reverse CAMP-reaction (Lämmler and Blobel, 1998; Almuzara et al., 2002; Ülbegi-Mohyla et al., 2009; Hijazin et al., 2010). PLD of *A. haemolyticum* was successfully cloned, sequenced and analyzed by Cuevas and Songer (1993), showing a putatitve signal sequence of 26 amino acids. Hence, a correlation of the coding regions of *A. haemolyticum* PLD displayed 65 % homology to PLD genes of both *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans*. A further molecular investigation of *A. haemolyticum* PLD pointed out to its pathogenic activity by enhancing restructuring of lipid rafts in plasma membranes of epithelial cells, an activity known to be involved in collaborating bacterial intrusiveness to host cells (Seveau et al., 2004; Abraham et al., 2005; Goluszko et al., 2008) and that could be repressed by applying anti-PLD antibodies or by cholesterol sequestration. Additionally, PLD augmented adhesion and

intrusion of bacteria and aggravated necrotic activities to invaded host cells (Lucas, 2009; Lucas et al., 2010).

Neuraminidases (sialidases) are widely distributed enzymes found in Eukaryotes, Prokaryotes and Archaea which belong to the glycosyl hydrolase enzyme group that splits off α2,3-linked sialic acids of various glycoproteins and carbohydrates into simpler carbon sources (Soong et al., 2006). Neuraminidases are recounted as key players in the adhesion process between bacteria and host epithelial cells. They are particularly found in bacteria inhabiting the upper respiratory tracts and act by decreasing the amount of mucus on membranes, thereby promoting adhesion and impingement into host cells (Jost et al., 2001; Galen et al., 1992). It was found that *A. haemolyticum* genome comprises two neuraminidase genes, known as NanH and NanA (Lucas, 2009). Analogous neuraminidases namely NanH and NanP, which play a similar role were also characterized in *T. pyogenes*, a recent species-member of genus *Arcanobacterium* (Jost et al., 2001; 2002).

The present study investigated an *A. haemolyticum* strain isolated from a purulent nasal discharge of a Poitou donkey (appendix 1), six strains recovered from six human patients from two hospitals in Copenhagen, Denmark (appendix 2) and the reference strain *A. haemolyticum* DSM 20595^T. Phenotypic analysis results revealed that all 7 strains and the reference strain *A. haemolyticum* DSM 20595^T produced, after incubation at 37 ºC for 48h in a candle jar, an enhanced zone of hemolysis on rabbit agar compared to a narrow zone of complete hemolysis on sheep blood agar, which is an indicatory property of *A. haemolyticum* (Lämmler and Blobel, 1988; Hassan et al., 2009; Ülbegi-Mohyla et al., 2009; Hijazin et al., 2010).

All seven strains and the reference strain *A. haemolyticum* DSM 20595^T cultivated on sheep blood agar displayed a synergistic CAMP-like hemolytic reaction with *S. agalactiae* and *R. equi* as indicator strains and a reverse CAMP reaction in the zone of staphylococcal β-hemolysin. The results were conclusive to *A. haemolyticum* strains previously described by various authors (Hassan et al., 2009; Ülbegi-Mohyla et al., 2009; Hijazin et al., 2010).

The biochemical characterization of all seven strains of the present study (appendix 1, 2) and reference strain *A. haemolyticum* DSM 20595^T using the commercial identification kit API-Coryne test 20 was coherent to the results of diverse authors (MacLean et al., 1946; Lämmler and Blobel, 1988; Ding and Lämmler, 1992; Lawson et al., 2001; Hoyles et al., 2002; Hassan et al., 2009; Lehnen et al., 2006; Hijazin et al., 2010). The API Coryne test proved to be a reliable system for the identification of *A. haemolyticum* (Freney et al., 1991; Gavin et al., 1992). Further biochemical enzyme activities were investigated using substrate-containing tablets, 4-methylumbeliferyl conjugated substrates and various other phenotypic tests. Based

on these tests, all seven *A. haemolyticum* strains investigated in the present study (appendix 1, 2) and the reference strain *A. haemolyticum* DSM 20595^T displayed positive activities with the enzymes pyrazinaminidase, alkaline phosphatase, β-D-galactosidase, α-D-glucosidase, and *N*-acetyl-*β*-glucosaminidase. Moreover, substrate utilization as sole carbon source could be obtained from D-glucose, D-ribose, D-maltose and D-lactose. Whereas, all seven strains of the present study and the reference strain exhibited no enzymatic activity for α-galactosidse, β-glucosidase, esculin hydrolysis, urease and gelatinase. In contrary to the reference strain *A. haemolyticum* DSM 20595^T, one of the seven strains showed no signs of nitrate reduction, five were negative for pyrrolidonyl arylamidase reaction and two were *β*-glucuronidase negative. Meanwhile, the enzymatic activity of α-mannosidase could be clearly observed in all seven *A. haemolyticum* strains of the present study and the reference strain, complying with the recommendations of Carlson and Kontianainen (1994) for *α*-mannosidase test as a rapid and simple tool for the identification of *A. haemolyticum.*

Furthermore, the *A. haemolyticum* strain from appendix1 isolated from a donkey, revealed moderate liquefaction of Loeffler medium compared to a negative reaction for the six *A. haemolyticum* strains of human origin from appendix 2 and the reference strain. These results generally adhere to the findings of Hassan et al. (2009) and Hijazin et al. (2010) for *A. haemolyticums* strains of equine origin. Meanwhile, the main cause of this liquefaction in *A. haemolyticum* isolated from equines compared to other origins still needs to be clarified. All seven *A. haemolyticum* strains of the present study and the reference strain *A. haemolyticum* displayed a negative reaction for catalase and caseinase and a positive reaction for DNase activities. Moreover, *A. haemolyticum* strain of appendix 1 and three of the six *A. haemolyticum* strains from appendix 2 displayed negative starch hydrolysis reaction in contrast to reference strain *A. haemolyticum* DSM 20595^T. A cross-reaction with streptococcal serogroup B-specific antiserum could be detected in all six strains of human origin of appendix 2 and the reference strain *A. haemolyticum* DSM 20595^T but not in *A. haemolyticum* strain of donkey origin from appendix 1, concurring with the views of Garcia-de-la-Fuente et al. (2012) and Brown et al. (2013), marking this property as a prevailing characteristic of *A. haemolyticum* from human origin.

MALDI-TOF MS analysis of the seven *A. hemolyticum* strains of the present study from appendixes 1, 2 were matched against 12 reference strains of genera *Arcanobacterium* and *Trueperella*. Antecedently, the strains were subcultured on sheep blood agar for 48 at 37 ºC under microaerobic conditions in a candle jar and consequently an additional protein extraction pre-analysis step was applied using Bruker ethanol formic acid method. It is suggested that this

step has a positive impact on the quantity and quality of generated spectral fingerprints (Alatoom et al., 2011), thus instigating a more precise analysis of the targeted strains (Bizzini et al., 2011). The studied strains were compared to an up-to-date reference library using the MALDI Biotyper version 3.1 software. Numerous reports had estimated the log (score) values for an analogous species to be at ≥ 2.0, while the log (score) values for the genus level falling between ≥ 1.7 and < 2.0. Hence, any log (score) values below 1.7 are considered inappropriate for identification (Mellmann et al., 2008; Barbuddhe et al., 2008; Ilina et al., 2009; Lartigue et al., 2009; Nagy et al., 2009). MALDI-TOF MS analysis of the present study (appendix 1, 2) allowed the identification of all seven strains to the species level matching against reference strain *A. haemolyticum* DSM 20595^T with log (score) values ≥ 2.0. According to various authors, MALDI-TOF MS analysis has emanated as a rapid and powerful tool for the species characterization of a broad spectrum of Gram-positive and Gram-negative bacteria up to the species level (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011). Moreover, MALDI-TOF MS analysis could successfully differentiate between various species of genera *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012b) for identification of *T. abortisuis* strains from porcine and bovine origin (Hijazin et al., 2012c), a *T. bernardiae* strain isolated from an anal swab of a three-day-old piglet (Hijazin et al., 2012a), a *T. pyogenes* strain recovered from a brain abscess of an adult roebuck and an *A. hippocoleae* strain isolated from a uterus swab of a healthy mare (Wickhorst et al., 2017a, b).

A molecular identification of the seven strains of the present study (appendix 1, 2) was carried out by amplification and sequencing of the 16S rDNA (Hassan et al. 2009) and glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap* using the newly designed gap primers of the present study (appendix 1). The seven strains of the present study and the reference strain *A. haemolyticum* DSM 20595^T were further characterized by amplification of an *A. haemolyticum* specific region of 16S-23S rDNA intergenic spacer region (ISR) and 23S rDNA described by Hijazin et al. (2010) and by the amplification of the genes encoding the putative virulence factors arcanolysin, phospholipase D, hemolysin A, CAMP factor family protein, collagen binding protein and neuraminidases A and H, by means of previously described oligonucleotide primers (Hassan et al., 2009; Hijazin et al., 2010) or latterly designed primers of the present study using sequences obtained from the *A. haemolyticum* complete genome (accession no: CP002045), submitted to GenBank by Yasawong et al. (2010). The aforementioned virulence properties of arcanolysin and phospholipase D were previously characterized by Jost et al. (2011) and Lucas et al. (2010), respectively. Meanwhile, a collagen binding protein and two diverse neuraminidase enzymes have also been precedently determined as potential virulence

factors in *T. pyogenes*, a former species of genus *Arcanobaterium* (Jost and Billington, 2005; Lucas, 2009) and are believed to be associated with pathogenic activities by involving in adherence, intrusion and colonization activities. According to the investigation of the potential virulence factors and the putative virulence genes, it was disclosed that all seven strains of the present study (appendix 1, 2) and the reference strain *A. haemolyticum* DSM 20595^{T} were positive for the genes encoding Arcanolysin, phospholipase D (PLD), CAMP factor family protein and hemolysin A. Most of these results concede with the assumptions of various authors for the prevlance of arcanolysin encoding gene *aln* (Jost et al., 2011), phospholipase D encoding gene *pld* (Lucas, 2009) and CAMP factor family protein encoding gene *cfa* (Hijazin, 2010) in all *A. haemolyticum* strains. However, the collagen binding protein encoding gene and Neuraminidase H encoding gene *nanH* could not be detected in the *A. haemolyticum* strain of appendix 1 but were present in two and four *A. haemolyticum* strains of appendix 2 of the present study, respectively. Whereas, Neuraminidase A encoding gene *nanA* was present in the *A. haemolyticum* strain of appendix 1 and four A. haemolyticum strains of appendix 2 of the present study. Whether the presence or absence of these potential virulence factors contribute to the virulence property of individual *A. haemolyticum* strains remains at present unclear.

According to Carlson et al. (1994), *A. haemolyticum* could be classified into two different biotypes which supervene distinctive morphological, hemolytic and pathogenic patterns. Smooth colonies acquire smooth edges, undergo average to high β-hemolysis and are more biased to cause wound infections. In contrast, rough colonies acquire rough, irregular-shaped colony edges which appear like a 'fried egg', undergo slight enhancement to no β-hemolytic activity and have more tendency to cause pharyngitis (Carlson et al., 1999).

The 73 *A. haemolyticum* strains studied by Ruther et al. (2015), were classified into smooth and rough colony biotypes and could be genetically distinguished at the arcanolysin locus. The strains included all seven strains from appendix 1, 2, two strains of horse origin (Hijazin, 2012) in addition to 63 strains of human origin and reference strain *A. haemolyticum* DSM 20595 (=ATCC9345). The strains were originated from different geographical regions (Finland, Denmark, Germany and USA) and were isolated from various types of pathogenic infections (sinusitis, pharyngitis, wound infections, abscesses and bacteremia). All 73 *A. haemolyticum* strains (Ruther et al., 2015) were from human origin except 3 that were isolated from a purulent nasal discharge of a Poitou donkey (appendix 1) and from infected wounds of two horses (Hijazin, 2012). To date, the only known technique to differentiate between *A. haemolyticum* smooth and rough biotypes is via the β-glucuronidase biochemical test, where smooth biotypes display negative β-glucuronidase activity compared to a positive reaction of rough biotypes

(Carlson et al., 1994). The study of Ruther and coauthors (2015) introduces a first molecular method for the differentiation between smooth and rough biotypes of *A. haemolyticum*. All 73 *A. haemolyticum* strains reported by Ruther and coauthors (2015) were cultivated on Todd-Hewitt (TH) agar plates supplemented with 6 % defibrinated horse blood in 5 % CO_2 for 48 hours which allowed visualization of smooth and rough colony morphology of the various *A. haemolyticum* strains in addition to the investigation of the hemolytic activity. Growth on TH agar could successfully classify the 73 *A. haemolyticum* strains into smooth biotype strains (n=36) and rough biotype strains (n=37). It has been also suggested from the phenotypic results that smooth colonies displayed a moderate to enhanced hemolysis on TH agar compared to a mild to no hemolysis of rough colonies. These results were conclusive for all 73 *A. haemolyticum* strains from Ruther et al. (2015), with a variation of a single highly enhanced hemolytic strain that has been apparently observed in the study as a rough biotype but was formerly marked as a smooth biotype by Carlson and coauthors (1994).

On the presumption that the diversification in hemolytic activity could be attributed to a disparity in the *aln*-coding regions within the various *A. haemolyticum* strains, *A. haemolyticum* reference strain DSM 20595^T (=ATCC 9345) had been selected as a smooth colony strain in addition to a randomly selected *A. haemolyticum* rough colony strain. According to the results from Ruther et al. (2015), the molecular amplification of gene *aln* of all *A. haemolyticum* strains of the present study revealed that all 36 smooth biotype strains yielded an expected 2.0 kb product, 21 rough isolates yielded also the 2.0 kb product but 16 rough isolates produced a 3.2 kb amplicon. Sequencing the complete 3.2 kb product exposed a 1.2 kb stretch insertion sequence (IS), located ~170 bp downstream from *aln* gene start codon that had 99 % nucleotide sequence identity to a transposase and integrase from IS*911* of *Corynebacterium diphtheriae*. Worth noting that the IS element detected in nearly half of the rough isolates was absent from all strains of American, Danish and German origin and were only present in Finish isolates, suggesting that acquiring this IS element could be accredited to geographical reasons. It was also found that the insertion of the IS element was positioned at the same loci in all the rough isolates. Referring to the observation of higher hemolysis activity in smooth colonies compared to mild to no hemolysis in rough colonies, it was notably seen that rough colony isolates bearing an IS element were relatively lower in hemolysis than IS element free rough colony isolates. However, this investigation remains inconclusive as the other rough colony isolates were also considered weakly hemolytic indicating that there might be other factors associated with this hemolytic activity.

Moreover, a PCR amplification of the *aln* intergenic region of all *A. haemolyticum* strains of the study of Ruther et al. (2015) yielded a 830 bp product which incorporates the 3' end of phosphoglycerate mutase, a tRNA, the Shine-Dalgarno (SD) sequence and the 5' end of *aln*. On further investigation of this upper *aln,* a ~40 nucleotide polymorphism region could be detected which allowed a further discriminative phylogenetic classification between smooth and rough colony isolates of *A. haemolyticum*. Notwithstanding some exceptions, this polymorphism could distinctively differentiate ~ 90 % of the strains into smooth and rough colonies. Furthermore, the strains could be differentiated based on the cleavage pattern of restriction enzymes. Hence, ~75 % of smooth colony isolates could successfully be cleaved by *Cla*I restriction enzyme, whereas, ~86 % of rough colony isolates were cleaved by *Xcm*I. This high correlation would consequently bring up the contribution of the upper stream *aln* region in the smooth and rough colony phenotypic property of *A. haemolyticum*.

In conclusion, the phenotypic analysis of the *A. haemolyticum* strains of appendix 1, 2 of this study, including comparative hemolysis on sheep and rabbit blood agar, CAMP-like reactions and reverse CAMP reactions and the API Coryne test conceded a fundamental identification of *A. haemolyticum*. Furthermore, MALDI-TOF MS analysis had appropriately identified all 7 *A. haemolyticum* strains of appendix 1, 2 up to the species level, highlighting the significance of this technique as a rapid and accurate diagnostic tool. Moreover, the genotypic identification of all strains of the present study (appendix 1, 2) could be approved by amplification and sequencing of the universal target regions 16S rRNA gene, 16S-23S intergenic spacer region (ISR) and the additionally investigated glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap.* The absence and presence of seven potential virulence factor encoding genes of the *A. haemolyticum* strains from appendix 1, 2, allowed a detailed characterization of the *A. haemolyticum* and might give evidence for differences in virulence properties of the strains. However, at present little is known about the pathogenic roles these potential virulence factors play in the species *A. haemolyticum*.

Regarding the *A. haemolyticum* strains of Ruther et al. (2015), it has been concluded that the polymorphism in the upstream region of arcanolysin encoding gene *aln* could be useful for the genetic characterization of *A. haemolyticum* into smooth and rough biotypes. It has also been suggested that an additional 1.2 kb *aln* segment (IS element) presented in nearly half of the investigated *A. haemolyticum* rough colonies might be attributed to geographical location as it has been only observed in the *A. haemolyticum* rough colony strains from Finland and not in the strains from the USA, Denmark or Germany. Furthermore, pertaining to the hemolytic activity of both smooth and rough colony strains of *A. haemolyticum*, it has been perceived that

smooth strains mainly exhibit high hemolysis activity compared to a low hemolysis activity in rough colony strains.

A brief outline of the *A. haemolyticum* strains of the present study (appendix 1, 2) along with two strains of horse origin (Hijazin, 2012) abstracted from the study of Ruther and coauthors (2015): Only three of the 9 *A. haemolyticum* strain were of the rough biotype (two of human origin and the donkey strain). Meanwhile, none of the strains whether of rough or smooth biotypes carried the 1.2 kb IS element within their *aln* coding region. All strain were cleaved with C*l*al but one strain of horse origin which could be neither cleaved with C*l*al nor *X*cml.

Notwithstanding the fact that all *A. haemolyticum* rough colony strains bearing the IS element within the *aln* coding region were relatively lower in hemolytic activity than the rough colony strains lacking this region, whether this IS element is directly associated with the hemolytic activity or there might be other supplementary factors incorporated remains unclear.

Phenotypic and genotypic characterization of *A. pluranimalium isolated from various animal origins*

The species *A. pluranimalium* was initially described by Lawson and coauthors (2001) for two strains recovered from the spleen of a dead harbor porpoise and a lung abscess of dead fallow deer. Nearly a decade later *A. pluranimalium* could be isolated in a mixed culture from a dog with pyoderma (Ülbegi-Mohyla et al., 2010), ovine specimens and from a single bovine mastitis milk sample (Foster and Hunt, 2011). More recently, *A. pluranimalium* from milk samples of cows with mastitis were further characterized by Moser et al. (2013) and Wickhorst et al. (2016).

According to Lawson et al. (2001) *A. pluranimalium* was phenotypically characterized as facultatively anerobic, straight to slightly curved, non-branching, Gram-positive, non-acid-fast and non-sporulating bacterium. *A. pluranimalium* was catalase-positive, showed α-hemolysis on blood agar and produced acid fermentation from glucose, ribose and maltose but not from glycogen, mannitol, lactose, saccharose or xylose.

In this study *A. pluranimalium* strains from various origins (n=15), (appendix 3, 4, 5) and the reference strains *A. pluranimalium* DSM 13483^{T} could be characterized phenotypically, by MALDI-TOF MS analysis and genotypically by sequencing the 16S rDNA, by amplification of the recently described *A. pluranimalium*-specific target gene *pla* and by various other target genes.

Phenotypically, all 15 *A. pluranimalium* strains of the present study isolated from various origins revealed a moderate hemolysis on sheep blood agar and a synergistic hemolytic reaction with staphylococcal *β*-hemolysin, *Rhodococcus equi* and *A. haemolyticum* as indicator strains. Additionally, using API Coryne test and various other biochemical tests, the strains displayed generally positive reactions with caseinase (n=10), catalase (n=9), hydrolysis of starch (n=13), *β*-glucuronidase (n=14) and generally negative reactions with *α*-glucosidase (n=14), *N*-acetyl-*β*-glucosaminidase (n=14) which were concurrent to previous results from Ülbegi and coauthors (2010a).

Referring to the MALDI-TOF MS analysis, all 15 strains could successfully be identified to the species level and would correspondingly cluster together with reference strain *A. pluranimalium* DSM 13483^T (appendix 4, figure 2; appendix 5, data not shown; appendix 6, figure 1). These results underpins the heretofore discussed MALDI-TOF MS analysis as an effective technique for the identification of a broad-spectrum of Gram-positive and Gram-negative bacteria up to the species level (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011).

All *A. pluranimalium* strains of the present study (n=15) (appendix 3, 4, 5) and the reference strain *A. pluranimalium* DSM 13483^T were characterized genotypically by amplification and sequencing of the 16S rRNA gene and the newly described pluranimaliumlysin encoding gene *pla*. Newly designed oligonucleotide primer for the amplification of gene *pla* could be achieved based on *pla* partial gene sequence obtained from GenBank (accession FR745890). Meanwhile, Moser and coauthors (2013) had concurrently and independently described gene *pla* as a novel target gene for molecular identification of *A. pluranimalium*. The sequences of the genes 16S rRNA and *pla* of the *A. pluranimalium* strains of the present study (n=13, n=1, n=1) (appendix 3, 4, 5) and the reference strain *A. pluranimalium* DSM 13483^T yielded a sequence identity ranging from 99.2 to 99.9 and from 98.9 to 99.6 % among each other, respectively. A nucleotide analysis of gene *pla* of *A. pluranimalium* with the corresponding genes *plo* of *T. pyogenes*, *phl* of *A. phocae*, *aln* of *A. haemolyticum* and the pore-forming toxin encoding genes *ply* of *Streptococcus pneumoniae*, *ily* of *Streptococcus intermedius* and *hly* of *Listeria monocytogenes* conceded a relatively higher correlation between the genes *pla*, *plo*, *phl* and *aln* (appendix 3, figure 2; appendix 4, figure 1; appendix 5, figure 2) indicating the close relation of these potential virulence factors of closely related species and genera. However, the role the pore-forming toxin pluranimaliumlysin plays in the process of infection remains unclear. Virulence properties of closely related pyolysin and arcanolysin have been previously described by Billington et al. (1997) and Lucas (2010).

Two *A. pluranimalium* strains of the present study (appendix 4, 5) were further characterized by amplification and sequencing of glyceradehyde 3-phosphate dehydrogenase encoding gene *gap* displaying a sequence identity of 99.1 % and 99.6 %, respectively to reference strain *A. pluranimalium* 13483^T. The *A. pluranimalium* strain isolated from a muskox (appendix 6) was additionally investigated by amplification and sequencing of the *β*-subunit of RNA polymerase encoding gene *rpoB* and the elongation factor tu encoding gene *tuf*. While the 16S rRNA gene sequencing continues to be the golden standard for the identification of a broad spectrum bacterial species (Claridge III, 2004; Janda and Abott, 2007; Chakravorty et al., 2010), the use of various housekeeping genes for a meticulous polyphasic taxonomy had been suggested by various authors (Maiden et al., 1998; Soler et al., 2004; Das et al., 2014). The *β*-subunit of bacterial RNA polymerase encoding gene *rpoB* had already been successfully used for the identification of *Staphylococus* species (Drancourt and Raoult, 2002), *Cornebacterium* species (Khamis et al. 2004) and the identification of genera *Enterococcus*, *Streptococcus*, *Gemella*, *Abiotrophia* and *Granulicatella* (Drancourt et al., 2004). It has also been used as a molecular marker in microbial ecological studies (Case et al., 2007) and was applied in REP-PCR and BOX-PCR for differentiating between various species of genus *Geobacillus* (Meintanis, 2008) and in RFLP for the identification of *Leptospira serovars* (Jung et al., 2015). Moreover, the elongation factor tu encoding gene *tuf* has been used as a successful diagnostic tool for the identification of the genera *Enterococcus*, *Streptococcus*, coagulase-negative *Staphylococcus* and *Lactococcus* (Li et al., 2012). The elongation factor tu encoding gene *tuf* has also been found useful in epidemiological typing studies such as in RFLP analysis for differentiation and classification of phytoplasms (Schneider et al., 1997) and for differentiation of lactic acid bacteria (Park et al., 2012). Both genes *rpoB* and *tuf* of the *A. pluranimalium* strain of the present study (appendix 5) displayed a sequence identity above 99 % with reference strain *A. pluranimalium* DSM 13483^T. The *A. pluranimalium* strain of appendix 6 could also be successfully identified using a *pla* LAMP assay (appendix 5, figure 5). Hence, comparable LAMP assays had been previously applied for the identification of *A. pluranimalium* isolated from bovine milk samples and from various origins (Abdulmawjood et al., 2015; Wickhorst et al., 2016), of *Leptospira* spp. (Koizumi et al., 2012 Lin et al., 2009), *Erysipelothrix rhusiopathiae* (Yamazaki et al., 2014), *Streptococcus equi* subsp. *zooepidemicus* (Kinoshita et al., 2014), *T. pyogenes* (Abdulmawjood et al., 2016) and ostrich meat (Abdulmawjood et al., 2014).

The phenotypic properties, the MALDI-TOF MS analysis and the genotypic properties investigating the 16S rRNA gene and genes *gap*, *rpoB* and *tuf* and the novel target gene *pla*

could successfully be used for identification of *A. pluranimalium*. This might help to identity the species *A. pluranimalium* in diagnostic laboratories in the future.

The *A. pluranimalium* strains of the present study were isolated from miscellaneous organs together with various other bacteria, indicating that the clinical significance of this species as a direct cause of disease in animals and possibly in humans remains to be elucidated.

However, recovering this species from various species of animals (i.e. porpoise, fallow deer, dog, sheep, cow, giraffe and muskox) emphasizes the name '*pluranimalium*' this species has been given.

Phenotypic and genotypic characteristics of the newly described species *A. canis* from dog and cat origin

Species of genus *Arcanobacterium* originally described by Collins et al. (1982b), represent, as already mentioned before, a group of facultative anaerobic, non-spore-forming, Gram-positive, rod-shaped bacteria. According to a proposal of Yassin and coauthors (2011) the genus was divided into two genera, retaining the species *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae* and transferring *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* and *A. pyogenes* to the new genus *Trueperella*.

The type strain *A. canis* DSM 25104^T (appendix 6) was isolated together with various other bacterial species during a routine diagnostic test from an otitis externa of a 7-year-old female bulldog.

A. canis was characterized as Gram-positive, oxidase-negative, non-motile, no-spore-forming rod-shaped bacterium that measured 1-2 µm in length and 0.5 µm in width. Upon growth on sheep blood agar, the strain displayed a weak zone of hemolysis under microaerobic conditions. CAMP-like reaction was positive with indicator strain *Rhodococcus equi*. Substrate utilization as a sole carbon source could be obtained from glucose, ribose, maltose, lactose, saccharose and glycogen but not from xylose or mannitol. A positive enzymatic activity in all *A. canis* strains could be observed with alkaline phosphatase, *β*-glucuronidase, *β*-galactosidase, *α*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase, DNase and amylase but no activity was encountered with nitrate reductase, pyrazinamidase, pyrrolidonyl arylamidase, aesculin hydrolysis, serolysis on Loeffler agar, urease, gelatinase, catalase, caesinase or hyaluronidase.

A phylogenetic analysis was performed by constructing a phylogenetic tree subsequent to a seed alignment of the 16S rRNA sequence of the type strain *A. canis* DSM 25104^T to the consonant 16S rRNA All-species living tree project (LTP), ARB database package 104, March

2011 (Ludwig et al., 2004). This analysis achieved by conjointly using the maximum-likelihood RAxML method, version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, the neighbor-joining method using the Jukes-Cantor model (Jukes and Cantor, 1969) and the maximum-parsimony method, DNAPARS version 3.6 (Felsenstein, 2005) (appendix 6, figure 1).

According to the neighbor-joining analysis, the 16S RNA gene sequence of strain *A. canis* DSM 25104^T displayed 99.2 % sequence similarity to an uncharacterized *Arcanobacterium* strain M214/96/1 and ranged from 95.8 % to 97.2 % sequence similarity with various other *Arcanobacterium* type strains and with *A. haemolyticum* CIP 103370^T as closest relative and from 93.9 % to 94.8 % sequence similarity with *Trueperella* type strains. The phylogenetic tree (appendix 6, figure 1) markedly revealed the clustering of the type strain *A. canis* DSM 25104^T within genus *Arcanobacterium* and could be discriminated from genus *Trueperella*. Implying to the 16S rRNA gene sequence signature nucleotides defined by Yassin et al. (2011), which discriminates genus *Arcanobacterium* from genus *Trueperella*, type strain *A. canis* DSM 25104^T revealed all 12 signature nucleotides explicit for genus *Arcanobacterium*.

Based on chemotaxonomical analysis the quinone content of type strain *A. canis* DSM 25104^T complied with the emended description of genus *Arcanobacterium* (Yassin et al., 2011) comprising 73 % MK-9(H_4) as a major menaquinone, 10 % MK-9(H_2), 8 % MK-9, 5 % MK-8(H_4), 1 % MK-8, 1 % MK-7(H_2) and 1 % MK-7. The polar lipid profile comprised predominantly from phosphatidylcholine, diphosphatidylglycerol which adheres to the genus *Arcanobacterium*, in addition to phosphatidylinositol mannoside and an unidentified phospholipid (PL2). It also revealed moderate amounts of phosphatidylinositol and minor amounts of various unidentified lipids.

Conforming to the fatty acid profile content of genus *Arcanobacterium* pedetermined by Yassin et al. (2011), the common prominent existing fatty acids were $C_{14:0}$, $C_{16:0}$, $C_{18:1}\omega 9c$ and $C_{18:2}\omega 6,9c$/anteiso-$C_{18:0}$, in addition to minor amounts of $C_{10:0}$ and $C_{12:0}$.

This species has been given the name '*A. canis*' referring to its original isolation from a dog.

The present study (appendix 7) describes the identification and characterization of four additional *A. canis* strains and the reference strain *A. canis* DSM 25104^T (appendix 6) by conventional methods, by MALDI-TOF MS analysis and genotypically by amplification and sequencing of the molecular target 16S rDNA, 16S-23S rDNA intergenic spacer region, 23S rDNA and the genes *rpoB* and *gap*.

Two strains of dog origin were recovered during routine microbiological diagnostic at the 'Institut für Hygiene und Infektionskrankheiten der Tiere' in Giessen, Germany, while the other

two strains (dog, n=1; cat, n=1) were isolated at the Veterinary Medical Teaching Hospital, Texas A & M University, Texas, USA. The four *A. canis* strains (appendix 7) were isolated together with various other pathogenic bacteria such as *Pseudomonas* spp., *Streptococcus canis*, *Staphylococcus aureus*, *Bacillus* spp. and *Corynebacterium* spp., *Escherichia coli* and *Entercoccus* spp.

A phenotypic investigation of the four strains (appendix 7), including hemolysis on sheep and rabbit blood agar, CAMP-like reaction, API Coryne test and various other biochemical tests showed a nearly equivalent outcome with reference strain *A. canis* DSM 25104^T (appendix 7, Table 1). MALDI-TOF MS analysis of the four *A. canis* strains exclusively conceded a correct identification of the four *A. canis* strains (appendix 7) to the species level with log (score) values ranging from 2.4 to 2.7 compared to reference strain *A. canis* DSM 25104^T but not when matched to the aforetime MALDI Biotyper 3.1 software package indicating that all four strains of the present study (appendix 7) belong to the newly described species *A. canis*.

The genotypic investigation of the four *A. canis* strains of this study (appendix 7) and reference strain *A. canis* DSM 25104^T by amplification and sequencing of the molecular targets 16S rDNA, 16S-23S rDNA intergenic spacer region (ISR), 23S rDNA and the genes *rpoB* and *gap* displayed sequence similarities ranging from 95.6 % to 97.1 %, 83.9 % to 87.2 %, 94.4 % to 98.7 %, 72.4 % to 76.8 % and 70.3 % to 78.9 % to various other species of genera *Arcanobacterium*, respectively. Moreover, the phylogenetic trees from appendix 7 (figures 2, 3) revealed that *A. canis* is prominently distinctive from other species of genus *Arcanobacterium*.

The newly described species *A. canis* (appendix 6, 7) could successfully be identified by phenotypic and biochemical properties, by MALDI-TOF MS fingerprinting analysis and by amplification and sequencing of various molecular targets proving the usefulness of these techniques in microbiological studies.

However, the *A. canis* strains of the present study from dog and cat origin were isolated from various infections together with diverse other potentially pathogenic bacteria indicating that their role in pathogenesis of diseases has yet to be elucidated.

Description and further characterization of *Arcanobacterium phocisimile* sp. nov., a marine bacterium isolated from harbor seals of the German North Sea

The present study describes and further investigates the novel species *A. phocisimile* (n=2; appendix 8) and three more *A. phocisimile* strains (appendix 9) isolated from harbor seals of

the German North Sea. All *A. phocisimile* strains (appendix 8, 9) were secluded from anal or vaginal swabs during a monitory program on free living harbor seals in the period between 2004 and 2007 except one strain (appendix 9) which was collectively recovered with *Bacillus* spp., *Enterococcus* spp., *Erysipelothrix rhusiopathiae* and α-haemolytic streptococci from a bronchopneumonic lung of a dead female seal (following necropsy).

The two *A. phocisimile* strains of the present study (appendix 8) were investigated by physiological and biochemical methods, by chemotaxonomic analysis of menaquinones, polar lipid profile and major fatty acids and additionally by various molecular differentiation techniques including DNA-DNA hybridization, 16S rRNA gene analysis and genomic fingerprinting analysis (BOX-PCR, ERIC-PCR & RAPD).

A. phocisimile could be characterized as non-motile, non-sporulating, Gram-positive, oxidase negative rod-shaped bacterium that displays a weak zone of hemolysis under microaerobic conditions compared to a less pronounced hemolysis zone under aeorobic and anaerobic conditions. A CAMP-like reaction could be detected with the indicator strains *Streptococcus agalactiae*, *Rhodococcus equi*, *Psychrobacter phenylpyruvicus* in addition to a reverse CAMP reaction with staphylococcal *β*-haemolysin. Observations of API Coryne test revealed an acid production from glucose, ribose, maltose, lactose, saccharose and glycogen but not from xylose or mannitol, in addition to a positive enzymatic activity for pyrazinamidase, *β*-galactosidase, *α*-glucosidase, *α*-mannosidase, catalase, DNase and amylase.

A chemotaxonomic analysis of the two *A. phocisimile* strains showed a quinone system consisting of MK-9(H_4) as a predominant compound in addition to minor amounts of MK-9 and trace amounts of MK-9(H_2) and MK-8(H_4). The polar lipid profile incorporated major amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosidase in addition to further unidentified glycolipids, phospholipids and aminolipids, whereas the major existing fatty acids were $C_{18:0}$, $C_{18:1}\omega 9c$, $C_{16:0}$ and summed feature 5 (comprising $C_{18:2}$ ω6*c*9*c* and/or anteiso-$C_{18:0}$). These results generally were in agreement with the emended description of genus *Arcanobacterium* (Yassin et al., 2011).

The DNA-DNA hybridization analysis, which is still considered the golden standard for species identification performed on the two strains (appendix 8) and reference strain *A. haemolyticum* DSM 20595^T as their closest relative, showed 82.5 % relatedness among the two strains (reciprocal value 99.0 %) compared to 59.1 % (66.5 %) and 64.7 % (49.1 %) between the two type strains and reference strain *A. haemolyticum* DSM 20595^T. Referring to the DDH recommended 70 % cut-off value, the two strains were considered a different species from *A. haemolyticum* and were assigned to the new species *A. phocisimile*.

A sequence alignment of a nearly full-length 16S rRNA gene of the two *A. phocisimile* strains (appendix 8) with members of genera *Arcanobacterium*, *Trueperella* and other members of the family *Actinomycetaceae*, as previously described in appendix 7, revealed a 100 % sequence identity among the two *A. phocisimile* strains, and a 95.5 % – 98.4 % sequence identity between the two strains and *A. haemolyticum* type strain CIP 103370^T as the closest relative. A further constructed phylogenetic tree plainly revealed a close clustering for both *A. phocisimile* strains within genus *Arcanbocterium* and a clear distance from genus *Trueperella* (appendix 8, figure 1). The aforementioned 12 signature nucleotides of the 16S rRNA gene specific for genus *Arcanobacterium* as described by Yassin and coauthors (2011), were all present in the two *A. phocisimile* strains (appendix 8). Meanwhile, a supplementary genotypic discrimination analysis between the two *A. phocisimile* strains and *A. haemolyticum* type strain as their closest relative using BOX-PCR, ERIC-PCR and RAPD analysis techniques could distinctively differentiate between the two strains as a unique species and type strain *A. haemolyticum* CIP 103370^T as a different species (appendix 8, figure 2).

In the present study (appendix 9) three additional *A. phocisimile* strains were characterized phenotypically and by MALDI-TOF MS analysis and compared to the two *A. phocisimile* strains from appendix 8 (reference strain *A. phocisimile* DSM 26142^T and a companion strain). All five *A. phocisimile* strains (appendix 9) were consequently investigated genotypically by amplification and sequencing the molecular targets 16S rDNA, 16S-23S rDNA intergenic spacer region and the genes *rpoB* and *gap*.

All three *A. phocisimile* strains (appendix 9) shared a similar phenotypic appearance to reference strain *A. phocisimile* DSM 26142^T and the companion strain (appendix 8). In contempt to the close phenotypical properties between the *A. phocisimile* strains and *A. phocae* (appendix 9, table 2), they could be differentiated through a positive pyrazinamidase reaction in *A. phocisimile* compared to a negative reaction with *A. phocae*. The analysis of MS spectra of the five *A. phocisimile* strains (appendix 9) showed no specific results using the Bruker data base. However, MALDI-TOF MS analysis could successfully identify all five *A. phocisimile* strains including reference strain *A. phocisimile* DSM 26142^T to the species level using the MALDI Biotyper 3.1 software package, displaying log (score) values ranging between 2.69 and 2.80, among each other. Comparing the resulting spectra to the MALDI-TOF MS spectra from various other species of genus *Arcanobacterium* (appendix 9, figure 1) served as an indication that all five strains belong to the newly described species *A. phocisimile*.

Comparing the 16S rDNA sequences of the *A. phocisimile* strains (appendix 9) to various other species of genus *Arcanobacterium* revealed a 100 % sequence identity among all five *A.*

phocisimile strains and a sequence similarity ranging from 95.4 % to 98.4 % to the other *Arcanobocterium* species with *A. haemolyticum* as closest relative (appendix 9, figure 2). An additional characterization by sequence analysis of 16S-23 rDNA intergenic spacer region and the molecular target genes *rpoB* and *gap* yielded sequence identities ranging from 99.4 % to 100 %, 99.8 % to 100 % and 99.9 % to 100 % among each other, respectively and from 82.4 % to 89.3 %, 69.0 % to 82.8 % and 73.2 % to 90.5 %, respectively, with various other species of genus *Arcanobacterium*.

The analysis of biochemical properties, peptidic spectra by MALDI-TOF MS and the sequences of the various molecular targets could reliably identify, characterize and differentiate all five *A. phocisimile* strains (appendix 9) from various other species of genus *Arcanobacterium*, including its closest relatives *A. phocae* and *A. haemolyticum*. While all *A. phocisimile* strains (appendix 8, 9) were collectively isolated with various other bacteria, partially from apparently healthy marine mammals, the role *A. phocisimile* plays in pathogenesis is still unknown.

Isolation and characterization of a second novel marine *Arcanobacterium* species, *Arcanobacterium pinnipediorum* sp. nov.

This novel species, based on a single bacterial strain (appendix 10) was isolated, like the other heretofore mentioned marine *Arcanobacterium* species (appendix 8, 9), during the routine microbial diagnostic on living harbor seals. The strain was collectively recovered in 2004 with several other bacteria (data not shown) from an anal swab of a living male harbor seal. The single strain (appendix 10) had a gram-positive, non-motile, non-spore-forming, pleomorphic, V-shaped rod appearance. The phenotypic investigation showed a weak hemolytic zone upon growth on sheep and rabbit blood agar. Colonies grew nearly 0.5 mm in diameter with a more pronounced growth under microaerobic conditions compared to a weaker growth under aerobic and anerobic conditions. Investigating the single *Arcanobacterium* strain phenotypically (appendix 10), revealed a positive CAMP-like reaction with the indicator strains *Streptococcus agalactiae* and *Rhodococcus equi*, as well as, a positive reverse CAMP reaction in the zone of staphylococcal *β*-hemolysin. An acid production could be clearly observed with glucose, maltose, lactose, xylose, saccharose and glycogen but not from ribose or mannitol. A further enzymatic activity could be detected for pyrrolidonyl arylamidase, pyrazinamidase, alkaline phosphatase, *β*-galactosidase, *α*-glucosidase, *N*-acetyl-*β*-glucosaminidase, catalase and

amylase but not for nitrate reductase, *β*-glucuronidase, *β*-glucosidase, *α*-mannosidase, aesculin hydrolysis, urease, gelatinase and serolysis on Loeffler agar or caseinase.

A chemotaxonomic analysis revealed a quinone system predominantly consisting of MK-9(H4) and minor amounts of MK-9 and MK-8(H4), a major component of the polar lipids: diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol manoside and a moderate to minor amount of five unidentified glycolipids, three unidentified aminolipids and five unidentified polar lipids. In addition, the major existing fatty acids were $C_{16:0}$, $C_{18:1}\omega 9c$, $C_{18:0}$ and summed feature 5 ($C_{18:2}\omega 6,9c$/anteiso-$C_{18:0}$) with minor amounts of $C_{14:0}$, $C_{12:0}$ and $C_{10:0}$. The peptidoglycan structure was of cross-linkage type A5α (L-Lys-L-Lys-D-Glu) (A11.51 according to www.peptidoglycan-types.info). The chemotaxonomic analysis results were in concurrence to the characteristics of genus *Arcanobacterium* as emended by Yassin and coauthors (2011).

Genotype-wise analysis was performed by partially sequencing the 16S rRNA gene and a detailed characterization using the ARB software package release 5.2 (Ludwig et al., 2004) and the database of the 'All-Species Living Tree' Project release LTPs111. The sequence was further aligned with SINA (v1.2.11) according to SILVA seed alignment (http://www.arb-silva.de; Pruesse et al., 2012) and assigned into the LTP database. The phylogenetic analysis was based on three constructed trees: a maximum-parsimony tree using DNAPARS v3.6, maximum-likelihood tree using RAxML v7.04 with GTR-GAMMA and a rapid bootstrap analysis and a neighbor-joining tree using the Jukes-Cantor correction as the evolutionary model with 100 resamplings. All calculations were based on the 16S rRNA gene sequences equivalent to the 132-1379 nt positions on *E. coli* (Brosius et al., 1978). A constructed tree based on these results (appendix 10, figure 1) revealed a monophyletic clustering of the single strain (appendix 10) within genus *Arcanobacterium* supported by a high bootstrap value. A pairwise sequence similarity analysis using the ARB neighbor-joining method displayed a higher 16S rRNA correlation for the single strain (appendix 10) with reference strains *A. phocae* and *A. phocisimile* as closest relatives, sharing a sequence similarity ranging from 98.4 % to 97.5 %, respectively. While the 16S rRNA sequence similarity with various other species of genus *Arcanobacterium* ranged between 95.3 % and 96.9 %. The single strain (appendix 10) incorporated all 16S rRNA gene sequence signature nucleotides previously defined by Yassin and coauthors (2011). A further DNA-DNA hybridization of the single strain (appendix 10) compared to the type strains *A. phocae* DSM 10002 and *A. phocisimile* DSM 26142^T as closest relatives displayed a percentage of 4.7 (reciprocal 56 %) and 23 % (reciprocal 7.7 %), respectively, below the cut-off value (70 %).

The genotypic, chemotaxonomic and physiological analysis of the single strain (appendix 10) isolated also from harbor seals could clearly differentiate and classify this strain as a novel *Arcanobacterium* species, given the name *A. pinnipediorum* originating from Pinnipedia, a group of semi-aquatic marine mammals.

Identification and characterization of the novel species *A. wilhelmae*, isolated from the vaginal discharge of a rhinoceros (*Rhinoceros unicornis*)

The present study investigates and describes a novel *Arcanobacterium* species that could be recovered from the vaginal discharge of Sani, a female asian rhinoceros (*Rhinoceros unicornis*) at the Wilhelma zoo in Stuttgart, Germany. According to the animal's medical record, Sani was an apparently healthy rhinoceros that no more displayed signs of heat following its fifth and last successful calving in 2008. Two years later, a clear yellow-beige muco-serous vaginal discharge could persistently be detected on the female rhinoceros. After a routine diagnostic test in 2012, the *Arcanobacterium* sp. nov. strain 647 (appendix 11) was collectively isolated in high numbers with streptococci of serological group L, *Streptococcus dysgalactiae* subsp. *equisimilis* and *Escherichia coli*. The 16S rRNA gene sequencing, MALDI-TOF MS analysis and Fourier transform Infrared (FTIR) spectroscopy, revealed that the same isolate was frequently recovered from vaginal swabs taken from Sani in 2013.

The phenotypic analysis revealed that *Arcanobacterium* strain 647 (appendix 11) was Gram-positive, oxidase negative, non-motile, asporogenic rods. Consistent to all other species of genus *Arcanobacterium*, strain 647 underwent an optimal growth on sheep blood agar at 37 ºC for 48 h under microaerobic conditions using a candle jar. Contrariwise, the strain responded negatively to the indicator strains known to produce a CAMP-like reaction with genus *Arcanobacterium*. Further phenotypic characterization of *Arcanobacterium* strain 647 (appendix 11) showed that substrate utilization as sole carbon source could be obtained from glucose, ribose, maltose, saccharose and glycogen but not from xylose, mannitol and lactose. *Arcanobacterium* strain 647 (appendix 11) exhibited an enzymatic activity for alkaline phosphatase, β-glucuronidase and α-glucosidase, whereas no enzyme activity was observed for nitrate reductase, pyrazinamidase, pyrrolidonyl arylamidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, aesculin hydrolysis, serolysis on Loeffler agar, urease, gelatinase and catalase.

A genotypic analysis of *Arcanobacterium* strain 647 (appendix 11) was performed using a nearly complete sequence of 16S rRNA gene. The analysis was carried out using the pairwise

sequence similarities to closest related type strains by EzTaxon (www.ezbiocloud.net; Kim et al., 2012). The phylogenetic trees were constructed with the help of ARB release 5.2 using the 'All species living tree project' database release LTPs119 (July, 23 2015). Additional sequences not existing in database were incorporated after alignment using the SILVA Incremental Aligner (SINA; v1.2.11). Alignments were manually scrutinized and gene sequences corresponding to *Escherichia coli* positions 128 to 1379 (Brosius et al., 1978) were included. The phylogenetic trees were calculated according to the maximum-parsimony, maximum-likelihood and neighbor-joining methods using DNAPARS v. 3.6, RAXML version 7.04 with GTR-GAMMA and rapid bootstrap analysis and ARB, respectively. The bootstrap analysis was based on 100 resamplings as described by Felsenstein (2005). Based on the 16S rRNA gene sequences using the aforementioned methods, *Arcanobacterium* strain 647 was more distinctly allocated to genus *Arcanobacterium* than *Trueperella* (appendix 11, figure 1) and clustering closely to reference strain *A. canis* DSM 25104^{T}. *Arcanobacterium* strain 647 shared 98.8 % 16S rRNA gene sequence similarity with *A. canis* DSM 25104^{T} followed by *A. phocisimile* (97.8 %), *A. phocae* (97.7 %), *A. haemolyticum* (97.4 %), *A. hippocoleae* (96.6 %), *A. pinnipediorum* (96.4 %) and *A. pluranimalium* (95.4 %). While the sequence similarities with type strains of members of genes Trueprella ranged between 94.2 % and 95.0 %.

Depending on 16S rRNA gene sequence correlation between *Arcanobacterium* strain 647 (appendix 11) and reference strain *A. canis* DSM 25104 ^{T}, a DNA-DNA hybridization analysis was carried out for both strains as described by Ziemke et al. (1998). The resulting low values 13.4 % (reciprocal 15.9 %) determined that the two strains were different species.

Additionally, the G + C content of the genomic DNA of strain 647 (appendix 11) and reference strain *A. canis* DSM 25104^{T} was accomplished using the DNA melting temperature method as described by Gonzales and Saiz-Jimenez (2002). The G + C content of genomic DNA of strain 647 was 58.7 mol % which was distinctively higher than the 51.1 mol % value for *A. canis* DSM 25104^{T}. However, this percentage value was also considered slightly higher than average range of 50 – 57 mol % for genus *Arcanboacterium*, as reported by Yassin et al. (2011).

Coinciding with genus *Arcanobacterium*, the quinones system contents of strain 647 (appendix 11) was found to be 71.3 % MK-9(H_4), 17.3 % MK-8(H_4), 8.4 % MK-9 and 2.9 % MK-9 (H_2). In like manner, the polar lipid delineation of strain 647 distinguished major mounts of diphosphatidylglycerol, phosphatidylcholine, moderate amounts of an unidentified phospholipid (PL1), phosphatidylinositol and phosphatidylinositol-mannosides with minor amounts of three unidentified phosphoglycolipids (PGL1-3) and one glycolipid (GL1).

The fatty acid profile of strain 647 incorporated a major camount of $C_{16:0}$ (32.1 %), $C_{18:0}$ (21.8 %), $C_{14:0}$ (19.5 %), $C_{18:1}$ $\omega 9c$ (18.5 %) and $C_{18:2}\omega 6,9c$/anteiso-$C_{18:0}$ (summed feature 5; 5.7 %) and a minor component of $C_{12:0}$ (2.5 %).

The peptidoglycan analysis displayed a cross-linkage type A5α ($_{L}$-Lys- $_{L}$-Ala-$_{L}$-Lys-$_{D}$-Glu) (A11.53) according to www.peptidoglycan-types.info.

The phenotypic, chemotaxnomic and genotypic analysis could clearly discriminate strain 647 as a novel species of genus *Arcanobacterium* that was given the name *A. wilhelmae*, from 'Wilhelma zoo' in Stuttgart, Germany, referring to the place the strain was originally isolated.

Chapter 5 - Summary

In the present study seven *A. haemolyticum* strains isolated from a donkey and human patients, 15 *A. pluranimalium* strains from ovine, bovine and giraffe origin and from a muskox and 12 strains from miscellaneous origins representing the four novel species *A. canis*, *A. phocisimile*, *A. pinnipediorum* and *A. wilhelmae* together with reference strains of the hitherto described nine species of genera *Arcanobacterium* and *Trueperella* were investigated.

The four novel species of genus *Arcanobacterium*, namely *A. canis*, *A. phocisimile*, *A. pinnipediorum* and *A. wilhelmae*, newly described in the present study, were isolated from a dog, from harbor seals and from a rhinoceros, respectively and were characterized based on a polyphasic taxonomic approach. This included a comparative 16S rRNA gene phylogenetic tree analysis, DNA-DNA hybridization, determination of DNA-based ratio and chemotaxonomical investigations by fatty acid, menaquinone and polar lipid composition analysis.

All 34 strains investigated in the present study, representing six different species of genus *Arcanobacterium*, were characterized phenotypically by traditional microbiological methods including morphological, physiological and biochemical properties and by MALDI-TOF MS analysis. A molecular DNA-based investigation was performed by 16S rDNA sequence analysis, by sequencing 16S-23S rDNA intergenic space region (ISR), 23S rDNA and the genes encoding the B-subunit of bacterial RNA polymerase (*rpoB*), glyceraldehyde 3-phosphate dehydrogenase (*gap*) and elongation factor tu (*tuf*).

The additionally performed PCR-mediated amplification of seven genes encoding the potential virulence factors aranolysin, phospholipase D, hemolysin A, CAMP factor family protein, collagen binding protein, neuraminidase A and neuraminidase H of the *A. haemolyticum* investigated in the present study allowed an individual strain characterization. Moreover, *A. pluranimalium* could additionally be characterized by the species-specific and newly described gene *pla* encoding pluranimaliumlysin. This might help to clarify the pathogenic role such putative virulence factors play in infections caused by these bacterial species.

A. haemolyticum is well known for its disease-causing role in humans, rarely in animals. However, *A. pluranimalium*, *A. canis*, *A. phocisimile*, *A. pinnipediorum* and *A. wilhelmae* were isolated in the present study as mixed culture with several other pathogenic and non-pathogenic bacteria indicating that the pathogenic importance of these species needs to be elucidated.

Chapter 6 - Zusammenfassung

In der vorliegenden Studie wurden sieben *A. haemolyticum*-Stämme, isoliert von einem Esel und Humanpatienten, 15 *A. pluranimalium*-Stämme, isoliert von Schafen, Rindern, einer Giraffe sowie einem Moschusochsen, 12 weitere Stämme verschiedener Herkunft mit Vertretern der vier neu beschriebenen Arten *A. canis*, *A. phocisimile*, *A. pinnipediorum* und *A. wilhelmae* zusammen mit den Referenzstämmen der bisher beschriebenen 9 *Arcanobacterium*- und *Trueperella*-Spezies untersucht.

Die vier Spezies der Gattung *Arcanobacterium*, nämlich *A. canis*, isoliert von einem Hund, *A. phocisimile* und *A. pinnipediorum*, isoliert von Seehunden sowie *A. wilhelmae*, isoliert von einem Nashorn, konnten in der vorliegenden Studie auf der Basis eines polyphasisch-taxonomischen Ansatzes als neue Arten beschrieben werden. Die Untersuchungen umfassten eine vergleichende phylogenetische Analyse des 16S rRNA-Gens, die DNA-DNA-Hybridisierung, die Bestimmung der DNA-basierten Basenverhältnisse und chemo-taxonomische Untersuchungen wie Fettsäure-, Menachinon- und polare Lipidanalysen.

Alle 34 untersuchten Stämme der Gattung *Arcanobacterium*, dies beinhaltete 6 unterschiedliche Spezies, wurden phänotypisch mit traditionellen mikrobiologischen Methoden, einschließlich morphologischer, physiologischer und biochemischer Eigenschaften, untersucht und zusätzlich mittels MALDI-TOF MS-Analysen charakterisiert. Die auf DNA-Ebene basierenden Untersuchungen umfassten eine 16S-rDNA-Sequenzanalyse, die Sequenzierungen der 16S-23S rDNA intergenic spacer-Region (ISR), des 23S rDNA-Gens sowie die Gene der B-Untereinheit der bakteriellen RNA-Polymerase, der Glycerinaldehyd-3-phosphatdehydrogenase und des Elongationsfaktors tu, *rpoB*, *gap* und *tuf*.

Die zusätzlich durchgeführte PCR-vermittelte Amplifikation von sieben Genen der potentiellen Virulenzfaktoren Arcanolysin, Phospholipase D, Hämolysin A, CAMP-Faktor-Family-Protein, Kollagenbindungsprotein, Neuraminidase A und Neuraminidase H der *A. haemolyticum*-Kulturen der vorliegenden Studie ermöglichte eine individuelle Stammcharakterisierung. Darüber hinaus konnte *A. pluranimalium* zusätzlich durch das speziesspezifische und neu beschriebene Pluranimaliumlysin kodierende Gen *pla* charakterisiert werden. Dies könnte helfen, die pathogene Bedeutung dieser mutmaßlichen Virulenzfaktoren bei bakteriellen Infektionen zu klären.

A. haemolyticum ist bekannt für seine krankheitsverursachende Rolle beim Menschen, seltener bei Tieren. Da die *A. pluranimalium, A. canis, A. phocisimile, A. pinnipediorum* und *A. wilhelmae* Kulturen aus Mischkulturen mit verschiedenen anderen pathogenen und

nichtpathogenen Bakterien isoliert wurden blieb die pathogene Bedeutung dieser Spezies bislang allerdings noch unklar.

Chapter 7 - References

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