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Quantification of Pseudomonas spp. in raw milk and molecular analysis of their proteolytic potential

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ABSTRACT

Psychrotolerant *Pseudomonas* spp. capable of secreting the heat-resistant peptidase AprX are one of the main reasons for premature spoilage of UHT milk or milk products. The AprX peptidase is encoded in the polycistronic *aprX-lipA2* operon, which varies considerably in its genetic organization among different pseudomonads, and multiple factors modulate the AprX expression. Thus, various *Pseudomonas* species differ significantly in their proteolytic potential, which substantially complicates the prediction of the AprX load in raw milk or the shelf life of derived UHT milk products on the basis of cell counts.

In order to identify all potential milk spoiling species, 185 type strains within the genus *Pseudomonas* were screened for the presence of the *aprX* gene, which is responsible for caseinolytic activity. In total, 43.8 % of the type strains tested carried *aprX,* and most milk-relevant isolates could be assigned to three of the 21 existing *Pseudomonas* subgroups, namely *Pseudomonas gessardii*, *Pseudomonas fluorescens* and *Pseudomonas fragi*. Furthermore, 22 different genetic organizations of the *aprX-lipA2* operon were identified, which differed mainly in the presence and localization of two putative autotransporter genes (*prtA*, *prtB*) and up to two lipase genes (*lipA1*, *lipA2*). Investigation of the correlation between operon type and proteolytic potential revealed higher enzyme activities for members of *P. gessardii* and *P. fluorescens* than *P. fragi*. The localization of *prtAB* within the operon, only found for representatives of *P. gessardii* and *P. fluorescens,* and its absence in *P. fragi* strains were particularly noticeable, indicating an important role of PrtAB in the AprX production.

To gain further insights into the regulatory influences of individual genes encoded in the *aprX-lipA2* operon as well as external factors on the AprX biosynthesis, the highly proteolytic strain *Pseudomonas proteolytica* WS 5128 was examined in more detail. For this purpose, the wild type and deletion mutants constructed in this work (*P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*) were cultivated in milk at different temperatures (12 °C and 30 °C) for comparative analysis of *aprX-lipA2* transcription, secreted AprX, and proteolytic activity. It was shown that AprX biosynthesis is most likely regulated at the transcriptional level by temperature, with low temperatures resulting in increased

mRNA levels. Moreover, reaching the early stationary phase resulted in an increased AprX production, presumably modulated by the Gac/Rsm system, at the translational level. Furthermore, first indications were obtained assuming translational regulation of the AprX biosynthesis by PrtA and PrtB. The lipase gene (*lipA2*), located at the distal end of the *aprX-lipA2* operon, seems important for mRNA stability, at least at high temperatures.

Finally, two triplex qPCR assays based on their proteolytic activity were developed to quantify seven proteolytic *Pseudomonas* species (*P. proteolytica*, *P. gessardii*, *Pseudomonas lundensis*, *Pseudomonas lactis*, *P. fluorescens*, *P. fragi*) commonly present in raw milk. Five species-specific probes with binding sites in *aprX* and a universal *Pseudomonas* probe targeting *rpoB* were designed. All six probes revealed a very high specificity and sensitivity and thus were suitable to detect and quantify pseudomonads in raw milk. Equivalent results were obtained when comparing the qPCR results using the universal *Pseudomonas* probe with cell counts from selective cultivation on CFC agar. Thus, the assay is a less time-consuming and more specific alternative to selective cultivation for the determination of *Pseudomonas* counts. Moreover, the analysis of 60 raw milk samples via the triplex qPCR assays revealed unique compositions of the *Pseudomonas* population, in which *P. lundensis*, *P. proteolytica*, and *P. gessardii* were most frequently detected. Therefore, the two triplex qPCR assays are a valuable tool to quantify the most common *Pseudomonas* spp. in raw milk and distinguish between high- and low-level proteolytic species. To assess the risk of peptidase production in raw milk in the future, thresholds defining a critical cell count, at which quality losses of UHT milk products are expected, need to be determined.

ZUSAMMENFASSUNG

Psychrotolerante *Pseudomonas* spp. welche die hitzeresistente Peptidase AprX sezernieren, sind einer der Hauptursachen für vorzeitigen Verderb von H-Milch und H-Milchprodukten. Die AprX-Peptidase ist Teil des polycistronischen *aprX-lipA2*-Operons, welches in seiner Genzusammensetzung zwischen verschiedenen Arten stark variiert, und dessen Expression durch zahlreiche Einflussfaktoren reguliert ist. Außerdem unterscheiden sich verschiedene *Pseudomonas* Spezies stark in ihrem proteolytischen Potenzial, was die Vorhersage der AprX-Belastung in Rohmilch oder Haltbarkeit der daraus hergestellten H-Milchprodukte anhand von Keimzahlen erheblich erschwert.

Es wurden zunächst alle potenziell Milch verderbenden *Pseudomonas* Spezies identifiziert, indem 185 Typstämme innerhalb der Gattung *Pseudomonas* auf die Anwesenheit des *aprX* Gens, welches die Grundvoraussetzung für caseinolytische Aktivität darstellt, untersucht wurden. Insgesamt besaßen 43.8 % der analysierten Typstämme das aprX Gen und die meisten Milch-relevanten Isolate konnten drei der insgesamt 21 existierenden *Pseudomonas* Untergruppen zugeordnet werden, nämlich *Pseudomonas gessardii*, *Pseudomonas fluorescens* und *Pseudomonas fragi*. Desweiteren wurden 22 verschiedene *aprX-lipA2* Operonstrukturen identifiziert, welche sich hauptsächlich durch die Anwesenheit und Position von zwei putativen Autotransportergenen (*prtA* und *prtB*) sowie maximal zweier Lipasegene (*lipA1* und *lipA2*) unterschieden. Bei der Untersuchung der Korrelation von Operonstruktur und proteolytischem Potential zeigte Vertreter von *P. gessardii* und *P. fluorescens* höhere proteolytische Aktivitäten als solche von *P. fragi*. Besonders auffällig war die Position von *prtAB* innerhalb des Operons, was nur bei Vertretern von *P. gessardii* und *P. fluorescens* zutraf, und das Fehlen in *P. fragi*-Stämmen, was auf eine Rolle von PrtAB bei der AprX-Produktion hinweist.

Um weitere Erkenntnisse hinsichtlich regulatorischer Einflüsse einzelner Gene, des *aprX-lipA2* Operons, sowie externer Faktoren auf die AprX Biosynthese zu erlangen, wurde der stark proteolytische Stamm *Pseudomonas proteolytica* WS 5128 näher betrachtet. Hierzu wurden der Wildtyp und konstruierte Deletionsmutanten (*P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*,

P. proteolytica Δ*lipA2*) bei verschiedenen Temperaturen (12 °C und 30 °C) in Milch kultiviert, um die *aprX-lipA2*-Operon-Gentranskription, die sekretierten AprX-Mengen und die proteolytische Aktivität vergleichend zu analysieren. Hierbei konnte gezeigt werden, dass die die AprX Biosynthese höchstwahrscheinlich auf transkriptioneller Ebene durch die Temperatur reguliert wird, wobei niedrige Temperaturen in einer erhöhten mRNA Menge resultierten. Außerdem führte das Erreichen der frühen stationären Phase zu einer erhöhten AprX Produktion, was vermutlich durch das Gac/Rsm System auf translationaler Ebene reguliert wurde. Zudem konnten erste Erkenntnisse gewonnen werden, welche eine Funktion von PrtA und PrtB auf translationaler Ebene der AprX Biosynthese vermuten lassen. Zusätzlich scheint das Lipasegen (*lipA2*) am distalen Ende des *aprX-lipA2* Operons für die mRNA Stabilität, zumindest bei hohen Temperaturen, wichtig zu sein.

Schließlich, wurden zwei Triplex qPCR Assays entwickelt, um sieben proteolytische *Pseudomonas* Spezies (*P. proteolytica*, *P. gessardii*, *Pseudomonas lundensis*, *Pseudomonas lactis*, *P. fluorescens*, *P. fragi*), die häufig in Rohmilch vorkommen, anhand ihres proteolytischen Potenzials zu quantifizieren. Dafür wurden fünf spezies-spezifische Sonden, mit Bindestellen im *aprX* Gen und eine allgemeine *Pseudomonas* Sonde mit einem Zielabschnitt in *rpoB* designet. Alle sechs Sonden zeigten eine sehr hohe Spezifität und sind durch ihre Sensitivität für den Nachweis von Pseudomonaden in Rohmilch geeignet. Beim Vergleich der qPCR-Ergebnisse mittels universeller *Pseudomonas*-Sonde mit Zellzahlen aus der selektiven Kultivierung auf CFC-Agar wurden gleichwertige Ergebnisse erzielt. Somit stellt der Assay eine zeitsparendere und spezifischere Alternative zur selektiven Kultivierung dar um die *Pseudomonas*-Zellzahlen zu bestimmen. Darüber hinaus ergab die Analyse von 60 Rohmilchproben mittels der Triplex-qPCR-Assays jeweils eine einzigartige Zusammensetzung der *Pseudomonas*-Population, in der P*. lundensis*, *P. proteolytica* und *P. gessardii* am häufigsten nachgewiesen wurden. Folglich sind die beiden Triplex-qPCR-Assays ein nützliches Werkzeug zur Quantifizierung der häufigsten *Pseudomonas* Arten in Rohmilch und zur Unterscheidung zwischen hoch und niedrig proteolytischen Spezies. Um das Risiko der Peptidaseproduktion in Rohmilch in Zukunft abschätzen zu können, müssen im nächsten Schritt Schwellenwerte bestimmt werden, die eine kritische Zellzahl definieren, bei der Qualitätsverluste von H-Milchprodukten zu erwarten sind.

ABBREVIATIONS

aa	amino acid
ANIb	average nucleotide identity based on BLAST
Ca^{2+}	calcium-ion
cfu	colony-forming units
CMP	caseinmakropeptide
ECF	extracytoplasmatic function
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	extracellular proteolytic activity
Fur	ferric uptake regulation
GGDC	genome-to-genome distance
HIT	high-temperature inactivation
HTI	high-temperature inactivation
LAB	lactic acid bacteria
LIT	low-temperature inactivation
LTI	low-temperature inactivation
MLSA	multilocus sequence alignment
NGS	next-generation sequencing
OPA	o-phthaldialdehyde
P.	*Pseudomonas*
PCC	total *Pseudomonas* cell count
QS	quorum sensing
SMUF	synthetic milk ultrafiltrate
TETRA	tetranucleotide usage patterns
TNBS	2,4,6-Trinitrobenzenesulfonic acid
UHT	ultra-high-temperature
Zn^{2+}	zink-ion

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I GENERAL INTRODUCTION

1 Raw milk microbiota and UHT milk production

Bovine raw milk is a highly nutritious growth medium for microorganisms because it is rich in proteins, carbohydrates, fat, vitamins, and minerals. It also has a nearly neutral pH of approx. 6.5 and high water activity (Hassan and Frank, 2011, Quigley et al., 2013). Initially, fresh raw milk is considered to be sterile when secreted by healthy cows' udder cells. Afterward, a rather complex and unique microbiota is formed through the introduction of microorganism from several contamination sources, e.g., teat canal and surface, milking equipment, feed, feces, air, and dust (Verdier-Metz et al., 2009, Hassan and Frank, 2011, Vacheyrou et al., 2011, Verdier-Metz et al., 2012). Consequently, a large diversity of bacteria is present in raw milk, including both, Gram-positive genera, e.g., *Bacillus*, *Microbacterium*, *Streptococcus*, *Lactobacillus*, *Carnobacterium,* and *Staphylococcus* as well as Gram-negatives, e.g., *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas, Serratia, Aeromonas,* and *Hafnia* (Champagne et al., 1994, Hantsis-Zacharov and Halpern, 2007, von Neubeck et al., 2015, Baur et al., 2015a). Some of these microorganisms, such as lactic acid bacteria (LAB), like *Streptococcus thermophilus*, *Lactococcus lactis*, *Levilactobacillus brevis* are harmless or even beneficial (Hugenholtz and Starrenburg, 1992, Michel and Martley, 2001, Leroy and De Vuyst, 2004, Carafa et al., 2019). Others are human pathogens like *Salmonella enterica*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Cronobacter* spp. or *Campylobacter* spp. (McAuley et al., 2014, Lahti et al., 2017, Artursson et al., 2018, Sonnier et al., 2018) or food spoiling bacteria causing quality flaws of processed dairy products, such as *Pseudomonas* spp., *Acinetobacter* spp. or *Bacillus* spp. (Gopal et al., 2015, von Neubeck et al., 2015, Baur et al., 2015a, Caldera et al., 2016).

In Germany, raw milk is not directly processed after milking but stored at refrigerated conditions (4-6 °C) for around one to two days at the farm (figure I.1) (De Jonghe et al., 2011, Vithanage et al., 2016, MIV, 2020). Consequently, low temperatures create an advancing environment for psychrotolerant bacteria, like *Pseudomonas, Lactococcus*, *Acinetobacter,* or *Streptococcus*, which overgrow mesophilic organisms, thus reducing the diversity of the raw milk microbiota considerably (Lafarge et al., 2004, Rasolofo et al.,

2010, De Jonghe et al., 2011, Fricker et al., 2011, Kable et al., 2016, Hahne et al., 2019, McHugh et al., 2020). The enumeration of psychrotolerant bacteria is often correlated with the secretion of several extracellular enzymes. For instance, *Acinetobacter* spp. were described to secrete mainly lipases, *Lactococcus* members were shown to exhibit mostly proteolytic activity, and *Pseudomonas* spp. secreted both peptidases and lipases (von Neubeck et al., 2015, Hahne et al., 2019). On the one hand, psychrotolerant bacteria's enzymes are functional even at low temperatures due to their structural flexibility (Siddiqui and Cavicchioli, 2006). On the other hand, their structure allows them to unfold reversibly during heating procedures like pasteurization and even ultra-high temperature (UHT) treatment and retain a proportion of their enzyme activity after subsequent cooling, which can cause quality defects in the final product. Thereby, UHT milk products are particularly at risk because, unlike ESL milk or pasteurized milk, they are stored at

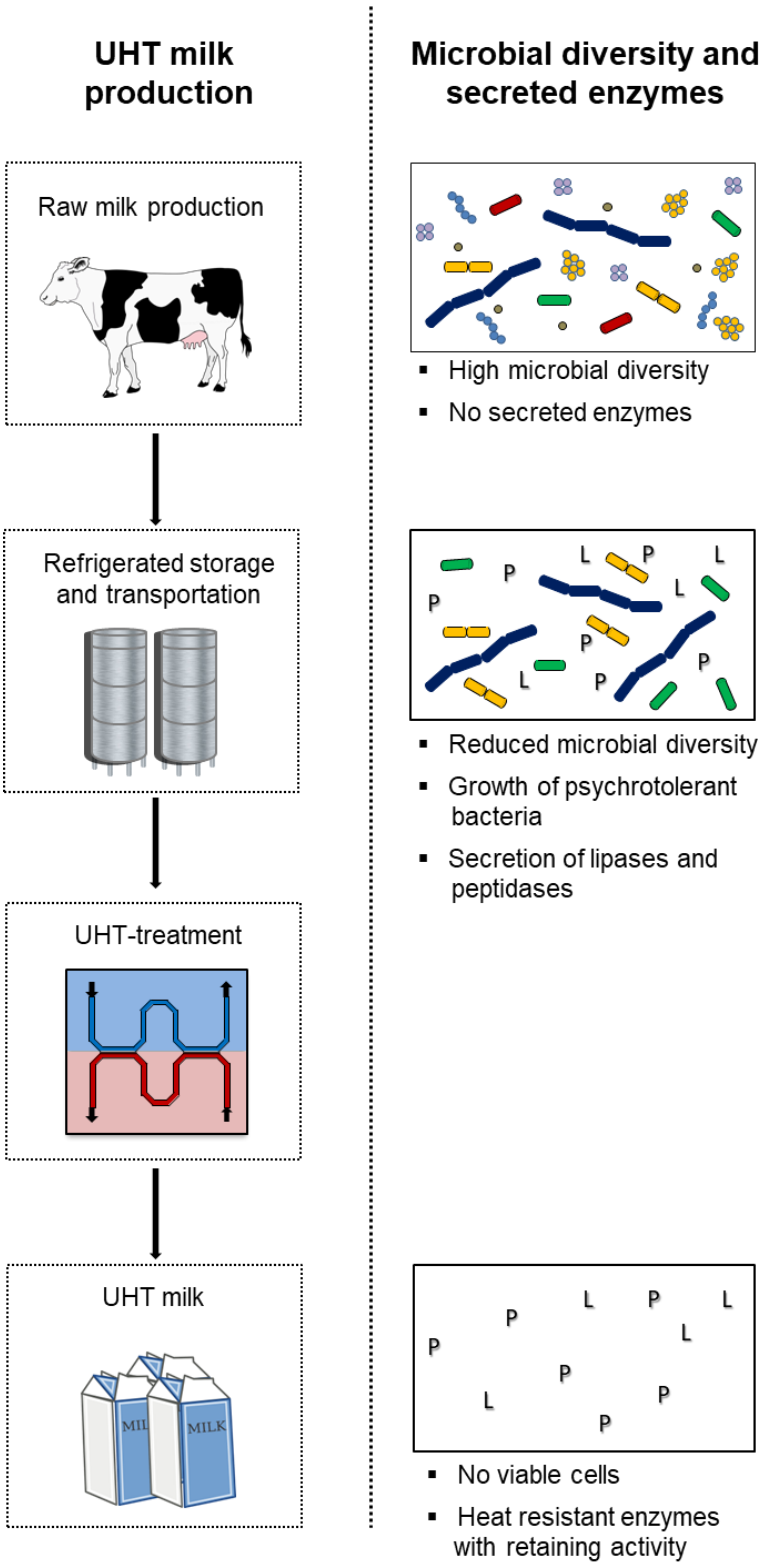

figure I 1: Schematic overview of the UHT milk production regarding the development of the microbial diversity in raw milk and occurrence of heat resistant peptidases (P) and lipases (L)

ambient temperatures over a long time and may not be consumed as quickly as other products. Whereas lipolytic degradation results in soapy flavor and rancidity, caseinolytic activity leads to bitterness, particle formation, and age gelation (Datta and Deeth, 2003, Deeth and Fitz-Gerald, 2006, Glück et al., 2016, Stoeckel et al., 2016a, Marchand et al., 2017).

Germany is the primary producer of dairy products in the European Union (European Commision, 2021). Since 1995 the production of raw milk has enlarged around 15 %. In 2019, approx. 50 % of in total 33.1 million tonnes of fabricated milk were produced for export, which is an increase of 29 % compared to 2018 (MIV, 2021). Thus, UHT-treated products are attractive for long-distance, non-refrigerated transport, especially to China, Arabia, or South America, due to their extended shelf life of approx. 4 - 6 months. All vegetative cells are destroyed during UHT treatment, applying a heating step between 135 °C - 150 °C for a few seconds (Scheldeman et al., 2006, Chavan et al., 2011). However, every year, heat-resistant enzymes cause quality defects in UHT-treated products leading to significant economic losses for the dairy industry. Consequently, even longer shelf life of up to one year at temperatures between 30 – 50 °C is desired (Stoeckel et al., 2016b). Nevertheless, extended heating periods or increased temperatures do not promise significant success to inactivate these enzymes sufficiently without negatively affecting the stability of milk components such as vitamin C or vitamin D or the product color (Asadullah et al., 2010, Chavan et al., 2011, Stoeckel et al., 2016b). Thus, the early detection of spoiling microorganisms, especially *Pseudomonas*, is of particular importance to ensure the quality of UHT milk products.

2 The genus *Pseudomonas* – a challenge for the food industry

The genus *Pseudomonas* is phylogenetically located in the family of *Pseudomonadaceae*, and contains, according to LSPN (April 2021), 253 validly described species. Thus, it includes the most representatives of all Gram-negative genera (Parte et al., 2020).

Historically, the phylogeny of *Pseudomonas* changed several times over the years, mainly due to the progressive development of applications used for classification. The genus *Pseudomonas* was first described by Migula in 1894. The name is composed of the Greek word 'pseudo', which means false, and 'monas', which originated probably from the nanoflagellate *Monas,* exhibiting similar cell shape and size (Migula, 1894,

Palleroni, 2010, Andreani and Fasolato, 2017). At the beginning of the 20th century, *Pseudomonas* was more like a pool for Gram-negative, rod-shaped, motile, and aerobic bacteria (Bergey et al. 1923). Through ribosomal RNA-DNA (rRNA-DNA) hybridization in the 1980s, *Pseudomonas* was subdivided into five distinct groups (rRNA group I-V), which were found to be genetically rather distant (Palleroni et al., 1973, Palleroni, 1984). Subsequently, members of the rRNA groups were redistributed to more than 25 other genera in the phylum of Proteobacteria (Woese, 1987, Kersters et al., 1996, Moore et al., 1996, Anzai et al., 2000). The *Pseudomonas*' sensu stricto' cluster (former rRNA group I) was included in the class γ-Proteobacteria, containing species genetically and phenotypically related to *Pseudomonas aeruginosa*, like *Pseudomonas fluorescens* or *Pseudomonas syringae* (Kersters et al., 1996, Moore et al., 1996, Peix et al., 2009).

Until today, 16S rDNA analysis is utilized to classify bacteria (Tindall et al., 2010). For *Pseudomonas,* however, 16S rDNA is not discriminative enough on the species level, which is why either alternative genes with higher resolution or multiple genes in a multilocus sequence analysis (MLSA) were required (Yamamoto et al., 2000, Tayeb et al., 2005). Therefore, housekeeping genes are well suited, as there are highly expressed and relatively conserved because they evolve faster than the *rrs* gene but slower than other protein-coding genes (Tayeb et al., 2005, Konstantinidis et al., 2006, Mulet et al., 2010). Mulet et al. performed an MLSA approach including 16S rDNA, *rpoB* (β-subunit of the RNA polymerase), *rpoD* (sigma factor binding RNA Polymerase), and *gyrD* (β-subunit of gyrase), which is reliable for *Pseudomonas* classification (Mulet et al., 2010).

The taxonomy of *Pseudomonas* will continue to change with broader use of next-generation sequencing (NGS) because it enables approaches such as tetranucleotide usage patterns (TETRA), genome-to-genome distance (GGDC), or average nucleotide identity (ANI), which allow a more subtle classification. However, Gomila et al. showed that the ANI based on BLAST (ANIb) approach exhibited a relatively high correlation to MLSA for the phylogeny of *Pseudomonas*. Thus, *Pseudomonas* is currently subdivided into three main lineages, *P. aeruginosa*, *P. fluorescens*, and *Pseudomonas pertucinogena* (Gomila et al., 2015, García-Valdés and Lalucat, 2016, Peix et al., 2018).

Pseudomonads possess a highly versatile metabolism, enabling them to adapt to an enormous amount of habitats (Stanier et al., 1966, Silby et al., 2011, Wu et al., 2011). *Pseudomonas* spp. have been isolated from water (Verhille et al., 1999, Manaia and

Moore, 2002, Zhong et al., 2015), soil (Weon et al., 2006, Ivanova et al., 2009, Lin et al., 2013), air (Sudharsanam et al., 2015), animals (Vodovar et al., 2005, Hameed et al., 2014, Menendez et al., 2015), plants (Gardan et al., 2002, Behrendt et al., 2003, Behrendt et al., 2009, Silby et al., 2011) and humans (Middleton et al., 2018, Vanderwoude et al., 2020). In general, members of *Pseudomonas* are relatively undemanding regarding nutrient requirements and can grow at a broad range of temperature (4°C - 42 °C) and between a pH of 4 - 8 (Moore et al., 2006). For instance, *Pseudomonas litoralis* or *Pseudomonas salina* can withstand high salt concentrations (Pascual et al., 2012, Zhong et al., 2015) or *Pseudomonas thermotolerans*, *Pseudomonas xinjiangensis*, or *Pseudomonas pelagia* extreme temperatures (Manaia and Moore, 2002, Hwang et al., 2009, Liu et al., 2009).

Pseudomonas spp. also includes several plant pathogens, like *Pseudomonas palleroniana*, *Pseudomonas syringae* or *Pseudomonas cichorii* (Gardan et al., 2002, Feil et al., 2005, Pauwelyn et al., 2011), animal pathogens such as *Pseudomonas entomophila* (Vodovar et al., 2005) and the opportunistic human pathogen *Pseudomonas aeruginos*a (Stover et al., 2000). Besides, some strains of *Pseudomonas putida* and *Pseudomonas fluorescens* were biotechnologically applied for biocontrol (Haas and Defago, 2005), and representatives of *Pseudomonas stutzeri*, *Pseudomonas tolaasii,* and *Pseudomonas veronii* were described to promote plant growth (Adhikari et al., 2001, Lami et al., 2020). Also, members of *P. stutzeri*, *Pseudomonas nitroreducens* were used for bioremediation (Bhatt et al., 2020, Coelho da Costa Waite et al., 2020), and strains of *P. aeruginosa* and *P. orientalis* were shown to produce secondary metabolites like antibiotics (Kerbauy et al., 2016, Simionato et al., 2017, Santos Kron et al., 2020).

However, due to their biological diversity, many pseudomonads exhibit various properties that present a real challenge for the food industry (figure I.2). Several species are psychrotolerant (von Neubeck et al., 2015, Caldera et al., 2016) and resistant to different antibiotics (Fanelli et al., 2021, Heir et al., 2021). Moreover, some members can produce pigments like pyoverdin or pyocyanin, leading to product color changes, e.g., in mozzarella cheese or fresh cheese (Martin et al., 2011, Caputo et al., 2015). As mentioned above, many *Pseudomonas* species can secrete several enzymes, including lipases, amylases, pectinolytic enzymes, and proteases, causing quality flaws like off-flavors or textural changes (Franzetti and Scarpellini, 2007, von Neubeck et al., 2015, Caldera et al., 2016). Besides, pseudomonads are susceptible to a broad range of

nutrients and able to produce biofilms, e.g., in pipes of milk-fabrication plants (Cherif-Antar et al., 2015, Liu et al., 2015, Radovanovic et al., 2020). On the one hand, biofilms present a challenge regarding the maintenance of hygiene. On the other hand, they can promote the establishment of multispecies biofilms, in which pathogens such as *Escherichia coli* or *Campylobacter jejuni* can immigrate and survive antimicrobial treatments, presenting health risks for consumers (Hilbert et al., 2010, Sternisa et al., 2019, Fanelli et al., 2021).

Concerning the affected foods, *Pseudomonas* spp. have been associated with the deterioration of seafood (Bohme et al., 2010, Dabade et al., 2015, Sternisa et al., 2019) poultry (Morales et al., 2016, Chmiel et al., 2020, Heir et al., 2021), meat (Franzetti and Scarpellini, 2007, Liu et al., 2015, Mohareb et al., 2015), vegetables, especially fresh-cut salad, (Franzetti and Scarpellini, 2007, Pauwelyn et al., 2011, Kahala et al., 2012, Pinto et al., 2015, Caldera et al., 2016) and dairy products such as UHT-milk or mozzarella cheese (Hantsis-Zacharov and Halpern, 2007, Ercolini et al., 2009, Marchand et al., 2009a, Marchand et al., 2009b, von Neubeck et al., 2015, Caldera et al., 2016, Stoeckel et al., 2016a).

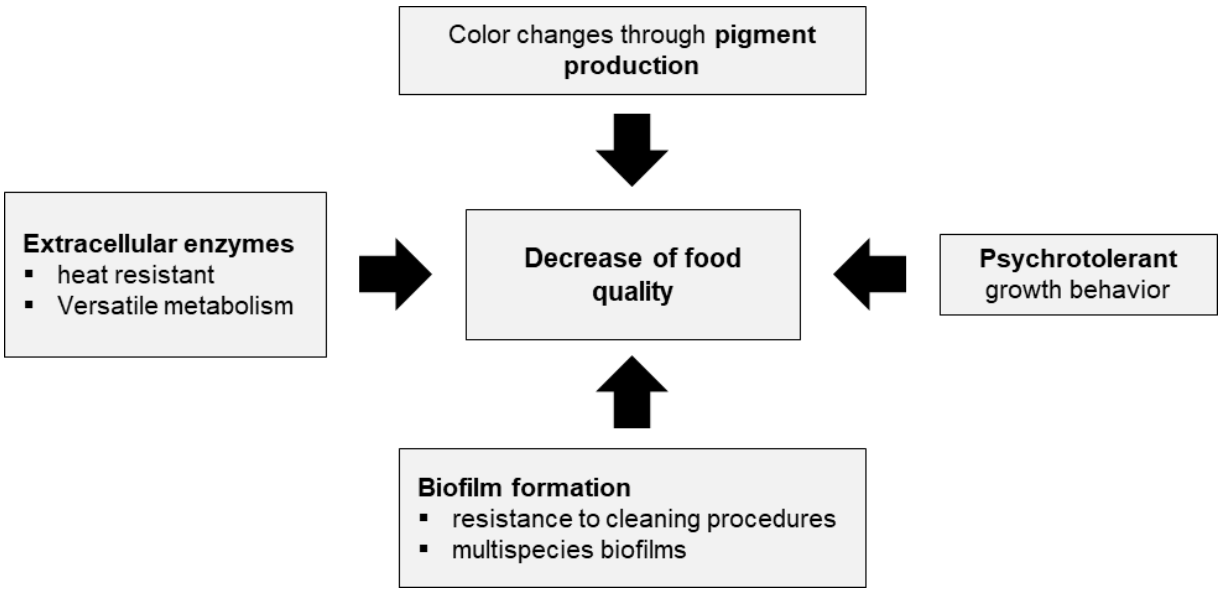

figure I 2: Characteristics of *Pseudomonas* species resulting in quality defects of food matrices.

Psychrotolerant pseudomonads are particularly challenging for UHT-milk and UHT milk products, as their heat-stable enzymes remain active after the UHT process and thus cause quality losses before reaching the minimum shelf life. On the one hand, pseudomonads are the dominant genus in raw milk, and on the other hand, they differ greatly in their prevalence. A study conducted by von Neubeck et al. depicted that in 20

analyzed raw milk samples 6 out of 33 *Pseudomonas* species made up more than 75 % of the isolates (von Neubeck et al., 2015). In general, species like *Pseudomonas proteolytica*, *Pseudomonas lundensis*, *Pseudomonas lactis*, *Pseudomonas meridiana*, *Pseudomonas protegens* and *Pseudomonas gessardii* were found more frequently in raw milk than ones like *Pseudomonas weihenstephanensis*, *Pseudomonas panacis* and *P. aeruginosa* (Marchand et al., 2009b, De Jonghe et al., 2011, von Neubeck et al., 2015, Baur et al., 2015a, Caldera et al., 2016, Glück et al., 2016).

3 The metallopeptidase AprX

Apart from the indigenous plasmin present in bovine milk, enzymes secreted by bacteria like *Acinetobacter*, *Pseudomonas*, *Chryseobacterium* or *Serratia*, are the leading cause of quality defects in UTH milk products. In particular, the occurrence of pseudomonads is frequently correlated with proteolytic degradation of milk casein.

Although *Pseudomonas* spp. are known to produce various extracellular proteases, only one caseinolytic peptidase (AprX) has been described so far, associated with milk decay (Liao and McCallus, 1998, Woods et al., 2001). AprX was first mentioned as a crucial virulence factor of *P. aeruginosa* PAO1 and was initially entitled Apr (**a**lkaline **pr**otease). In the following, the designation of AprX proved to be misleading. Whereas two different enzymes, namely AprA (former Apr) and AprX, were described in *P. aeruginosa* PAO1, in *P. fluorescens* B52*,* only AprX was characterized. However, AprA from *P. aeruginosa* and AprX from *P. fluorescens* were shown to be homologous, caseinolytic peptidases, while the function of AprX in *P. aeruginosa* remains unknown (Guzzo et al., 1991, Liao and McCallus, 1998, Duong et al., 2001, Woods et al., 2001, Ma et al., 2003). In the context of proteolytic milk decay, AprX and AprA are used as synonyms, and AprX has become the most widely accepted term in literature (Duong et al., 2001, Woods et al., 2001, Matéos et al., 2015, Zhang et al., 2018).

Among *Pseudomonas* species, the sequence of *aprX* is very heterogeneous, ranging from 63 – 99 % nucleotide sequence similarity (Kumeta et al., 1999, Marchand et al., 2009b). AprX is a member of the M10B subfamily (serralysin, EC 3.4.24.40), which is part of the metallopeptidase (MA) clan (Rawlings et al., 2018). Serralysins are widely distributed among Gram-negative bacteria (Cerda-Costa and Gomis-Ruth, 2014). Thus, AprX shares approx. 50 - 55 % amino acids (aa) sequence similarity with Ser2 found in *Serratia* and with proteases from *Erwinia* entitled PrtA, PrtB, PrtC (Liao and McCallus,

1998). Consequently, it is not surprising that these homologs share structural features and biochemical properties with AprX (Kumeta et al., 1999, Marchand et al., 2009b, Rawlings et al., 2018, Alves et al., 2019).

3.1 Characteristics of the AprX metallopeptidase

Depending on the respective *Pseudomonas* species, AprX has a size between 45 – 50 kDa (Dufour et al., 2008, Marchand et al., 2009b, Matéos et al., 2015). The catalytic center is formed by the N-terminal domain, which includes a binding motif (HEXXHXUGUXH) with three histidine residues anchoring a Zn^{2+} ion and a methionine residue that forms a Met-turn, necessary for the structure of the active center. Besides, the cleavage reaction in the catalytic cleft is enhanced by an additional glutamate residue providing a hydrophobic environment (Cerda-Costa and Gomis-Ruth, 2014, Ertan et al., 2015, Matéos et al., 2015, Rawlings et al., 2018, Alves et al., 2019). The C-terminal domain forms parallel β-sheets containing multiple glycine-rich repeats (GGXGXDXUX), able to bind six to eight Ca^{2+} ions, which increases the AprX stability (Feller, 1996, Kumeta et al., 1999, Dufour et al., 2008, Ertan et al., 2015, Matéos et al., 2015). A high proportion of hydrophobic amino acids and the absence of cysteine residues (meaning no formation of S-S bridges) result in high flexibility, and consequently, in a large thermostability.

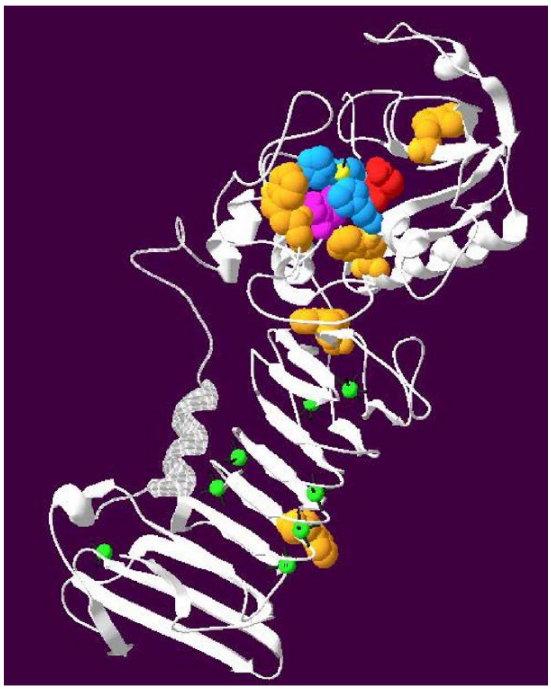

figure I 3: Protein structure of AprX from *P. aeruginosa*. In the catalytic site histidine residues are indicated in blue, glutamate in red, methionine in magenta and the Zn^{2+} ion in yellow. In addition, tryptophan (orange) and stabilizing Ca2+ ions (green) are depicted (Ertan et al., 2015).

The conformational change of the AprX structure depends on temperature and is roughly subdivided into three different states, the active state, the low-temperature inactivation state (LTI) and the high-temperature inactivation state (HTI), which all transit smoothly into each other (reviewed by Stoeckel et al. 2016b). Through the high flexibility, AprX is already active at low temperatures around 5 °C, which is typical for enzymes secreted

by psychrotolerant bacteria (Ertan et al., 2015, Matéos et al., 2015). Thus, AprX begins to unfold early, even before reaching the maximum turnover rates between 35 - 45 °C (Hamamoto et al., 1994, Kumeta et al., 1999, Dufour et al., 2008, Ertan et al., 2015, Glück et al., 2016, Stoeckel et al., 2016b). Consequently, whereas the amount of unfolded peptidases increases, the number of native enzymes decreases with rising temperatures. However, as native peptidases are still active, they degrade unfolded enzymes (autolysis). This process occurs in LTI and is irreversible. In LTI the highest degree of degradation is reached between 50 °C and 60 °C. The ratio is developing in favor of the unfolded enzymes through further temperature elevation, causing a reduction of autolysis. Interestingly, LTI is strongly attenuated in milk by the presence of calcium ions, which impede the conformation change of AprX, and casein, which competes with the unfolded peptidases as a substrate (Barach et al., 1978, Schokker and van Boekel, 1998, Glück et al., 2016). HTI above 95 °C causes a complete but reversible inactivation of the enzymes. During subsequent cooling, AprX was shown to return partially back to the active form, causing quality defects even though the product was ultra-high temperature treated (Marchand et al., 2009b, Ertan et al., 2015). The residual activity of AprX depends on the individual species and the growth medium. For instance, peptidases isolated from *P. lactis*, *Pseudomonas paralactis* and *P. weihenstephanensis* retained at least 45% of their activity after UHT treatment (138 °C, 18 s) in synthetic milk ultrafiltrate (SMUF) medium (Glück et al., 2016), while *P. paralactis* revealed 88% residual activity in whole milk after the same heating process (Baur et al., 2015b).

In whole milk, casein accounts for approx. 77 % of the total milk protein in bovine raw milk and is distinguished according to the amino acid (aa) composition into κ-, β-, α_{S1}- and α_{S2}-casein. Whereas β-, α_{S1}- and α_{S2}-casein are mainly located within the casein micelles, κ-casein forms through the hydrophilic part a "hairy structure" at the surface, which is essential for steric repulsion between casein micelles (Stenzel, 2016). AprX peptidase hydrolyzes preferentially κ-casein, in particular, in the f(105-125) region (Bagliniere et al., 2012). Thus the steric repulsion between micelles is considerably reduced, causing their aggregation (Datta and Deeth, 2003). Moreover, AprX non-specifically cleaves β-casein, except for the N-terminal part, which is difficult to access for the peptidase due to several phosphoseryl residues. Besides, α_{S1}- and α_{S2}-casein are hydrolyzed only in very few sites (Bagliniere et al., 2012). Regarding the appearance of quality defects in milk products, casein degradation results initially in sensory defects, followed by particle formation and finally gelation (figure I 4). The occurrence of quality

flaws, depends mainly on the apparent proteolytic activity and the storage time (Datta and Deeth, 2003, Stoeckel et al., 2016a, Zhang et al., 2018).

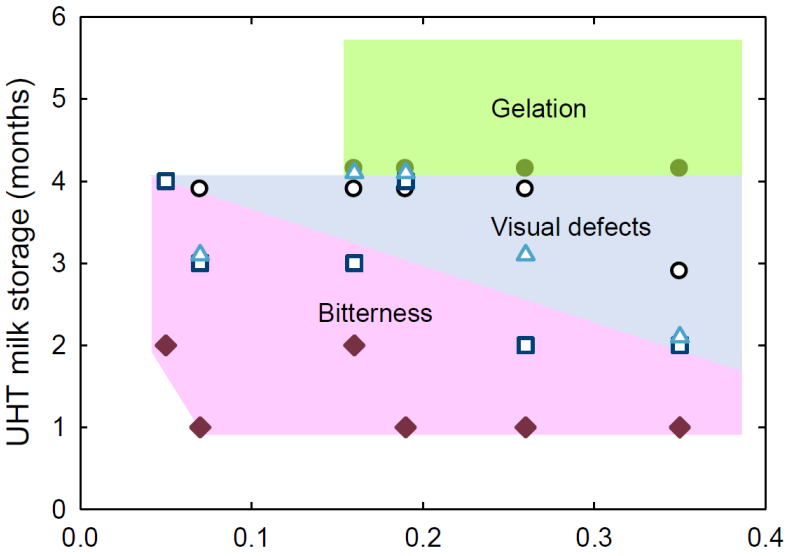

figure I 4: Appearance of quality defects in UHT milk in relation to apparent AprX activity and storage time (Stoeckel et al., 2016a)

However, the milk spoilage also depends on the proteolytic activity which was shown to be highly species- and partly even strain-specific (Bagliniere et al., 2012, von Neubeck et al., 2015, Baur et al., 2015a, Caldera et al., 2016). Bagliniere et al. assigned different *P. fluorescens* strains to two distinct groups, depending on their hydrolysis pattern of casein. Whereas group 1 strains were highly caseinolytic, strains of group 2 did not reveal any cleavage products during incubation of 90 days (Bagliniere et al., 2012). Furthermore, Cladera et al. observed generally elevated proteolytic activities for representatives of *Pseudomonas gessardii* and *P. proteolytica* compared to *Pseudomonas fragi*, which is in concordance with findings of von Neubeck et al. (von Neubeck et al., 2015, Caldera et al., 2016). Due to the differences in proteolytic potential, the composition of the *Pseudomonas* population in raw milk is crucial for the spoilage probability of the milk products made from it.

3.2 Genetic composition of the *aprX-lipA2* operon

In most *Pseudomonas* species secreting the AprX peptidase, *aprX* is the first gene of the polycistronic *aprX-lipA2* operon (Kawai et al., 1999, Woods et al., 2001, Ma et al., 2003). The *aprX-lipA2* operon comprises up to 9 different genes (figure I.5) and can be with some exception divided in a conserved (*aprXIDEF*) part and a region with variable gene composition (*prtAB lipA2/lipA1*) (Kawai et al., 1999, Duong et al., 2001, Woods et al., 2001, Ma et al., 2003). The conserved region includes genes encoding, besides AprX, a peptidase inhibitor (AprI), operating as degradation protection of periplasmic proteins (Bardoel et al., 2012), and a Type I secretion system (AprDEF) (Ma et al., 2003, Bardoel

et al., 2012). AprDEF was shown to be an ABC-transporter, secreting both AprX and the lipase LipA2, independent of a specific transport signal (Duong et al., 1994, Liao and McCallus, 1998). It consists of an inner membrane protein (AprD), a membrane fusion protein (AprE), and an outer membrane protein (AprF) (Ahn et al., 1999, Kawai et al., 1999, Woods et al., 2001).

The variable region can comprise genes for two putative autotransporters (PrtA and PrtB, also entitled PspA and PspB) with homologies to serine proteases and up to two lipases (LipA1 and LipA2). It was shown that the *aprX-lipA2* operon of *P. fluorescens* Pf0-1 includes an additional lipasegene (*lipA1*) downstream of *lipA2*, *Pseudomonas brassicacearum* NFM421 lacks *prtA* and *P. fluorescens* SIK W1 *prtA* and *prtB*. Remarkably, *P. aeruginosa* exhibits an entirely different operon structure, including a significant variation of the conserved region (Duong et al., 2001, Ma et al., 2003).

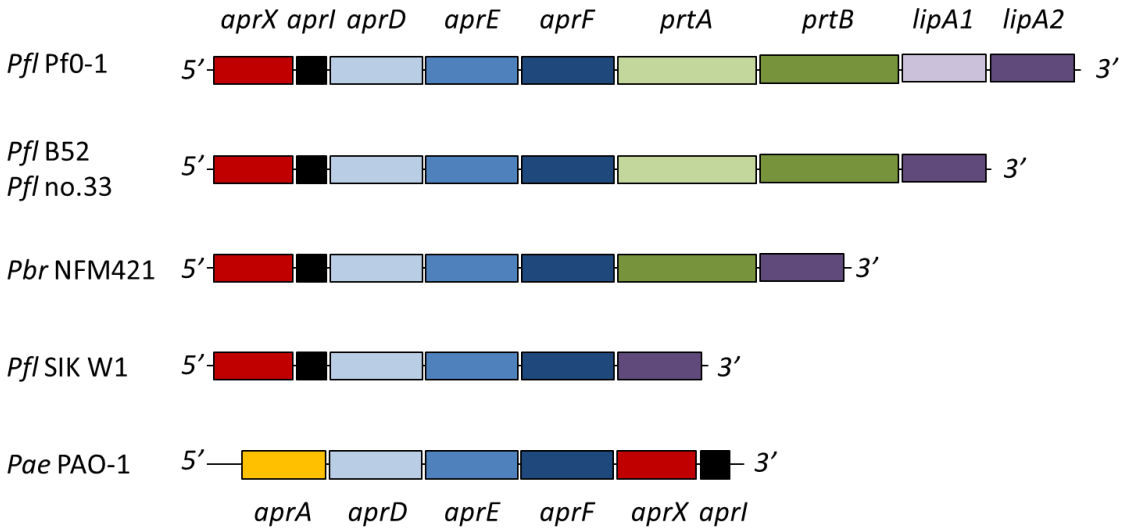

figure I 5: Genetic structures of the *aprX-lipA2* operon in different representative of *P. fluorescens* (*Pfl*), *P. brassicacearum* (*Pbr*) and *P. aeruginosa* (*Pae*). Figure adapted from Fig 1 of Ma et al. (2003).

3.3 Regulation of the *aprX-lipA2* operon and the proteolytic activity of AprX

On the one hand, the *aprX* gene is the basic requirement for *Pseudomonas* spp. to be proteolytic, but it does not necessarily assure caseinolytic behavior. Other genetic factors like differences in the substrate turnover rate are decisive for the intensity of the proteolytic activity. For example, Glück et al. demonstrated that the AprX peptidase, isolated from *P. weihenstephanensis* WS 4993, exhibited a lower substrate turnover rate

than those from *P. lactis* WS 4992 and *P. paralactis* WS 4672 (Glück et al., 2016). On the other hand, environmental factors such as cultivation temperature or iron concentration were shown to modulate the AprX biosynthesis of *P. fluorescens* B52 at different regulatory levels (Woods et al., 2001). Accordingly, a rather complex regulation of the *aprX* expression, including multiple factors, can be assumed for pseudomonads. However, only little is known about the underlying regulatory mechanisms.

For different strains of *P. fluorescens* and *P. chlororaphis* several studies determined an increased proteolytic activity between late exponential and early stationary growth phase (idiophase), indicating an influence of the cell density on the *aprX-lipA2* operon expression (Kohlmann et al., 1991, Nicodeme et al., 2005, Dufour et al., 2008, Alves et al., 2018). Thus, a modulation through the Gac/Rsm signaling cascade was assumed, also activated at the idiophase (Heeb and Haas, 2001). In many γ–proteobacteria, including *Pseudomonas*, the Gac/Rsm signal cascade or their homologs are highly conserved. Gac/Rsm is a global expression regulator of proteins involved in motility, biofilm formation, energy metabolism, production of secondary metabolites, and extracellular enzymes (Lapouge et al., 2008, Sobrero and Valverde, 2020). The two-component system GacS/GacA (**g**lobal activation of **a**ntibiotic and **c**yanide synthesis) acts as the starting point of the signal cascade (figure I 6). a yet unknown signal, associated with high cell densities, leads to the autophosphorylation of the membrane-bound sensor kinase (GacS), which activates the response regulator (GacA) through a phosphorelay mechanism (Heeb et al., 2002, Zuber et al., 2003, Kay et al., 2005, Goodman et al., 2009). GacA promotes the transcription of several small RNAs (sRNA), including RsmY, RsmX, and RsmZ. These sRNAs lead to the relief of translation repression, which is caused by mRNA binding proteins, RsmE and RsmA (Heeb et al., 2002, Valverde et al., 2003, Kay et al., 2005, Reimmann et al., 2005, Hassan et al., 2010, Cheng et al., 2013). In the absence of the regulatory sRNAs, RsmA/E hinders the ribosome from accessing the Shine-Dalgarno sequence to initiate mRNA translation of different mRNAs, including the *aprX-lipA2* operon (Lapouge et al., 2008).

A mechanism related to the GacS/GacA system and possibly affecting the *aprX-lipA2* expression is phase variation (van den Broek et al., 2005a). Phase variation is widespread among pseudomonads and serves to colonize new habitats or overcome nutrient deficiency rapidly. Thereby, secondary metabolism and synthesis of extracellular enzymes are modified by forming heterogeneous phenotypes (Cutri et al., 1984,

Chabeaud et al., 2001, Bartoli et al., 2014). Studies revealed that this mechanism can be triggered either by epigenetic modifications in gene expression, slipped strand mismatch through sequence repeats, recombinases, or spontaneous mutation (van den Broek et al., 2005a, 2005b).

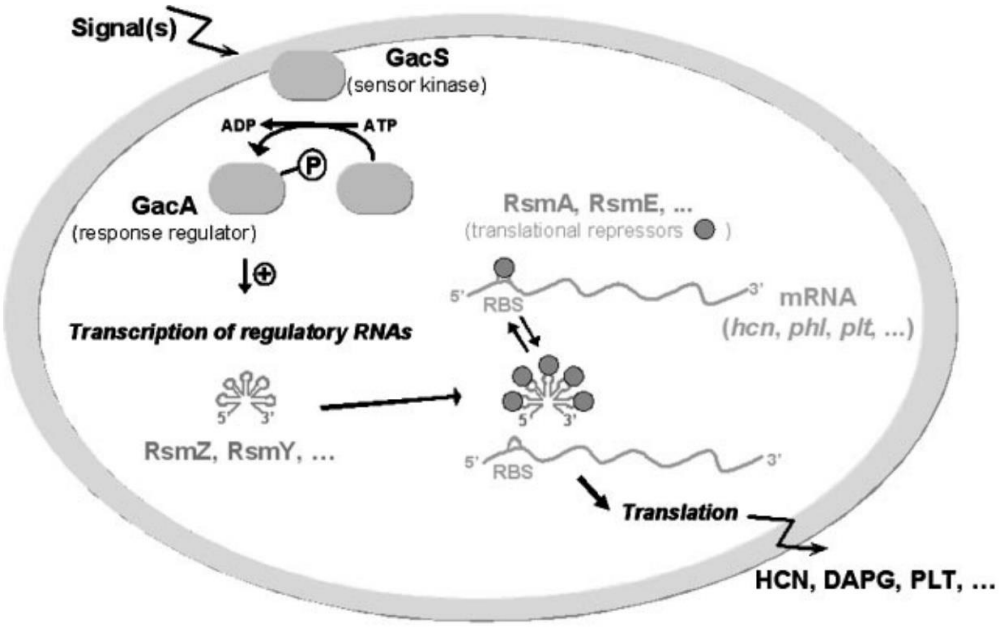

figure I 6: Principle of the Gac/Rsm signal transduction cascade (Haas et al., 2003)

Temperature is another major factor influencing the expression of the *aprX-lipA2* operon and the proteolytic activity in various *Pseudomonas* spp. (McKellar and Cholette, 1987, Burger et al., 2000, Humair et al., 2009, Baur et al., 2015a, Alves et al., 2018). On the one hand, the substrate turnover rates of the AprX peptidase isolated from members of *P. panacis*, *P. lactis*, *P. paralactis,* and *P. weihenstephanensis* increase with rising temperature and reach their maximum between 35 °C and 40 °C (Baur et al., 2015b, Glück et al., 2016). On the other hand, the growth rate of cold-adapted *Pseudomonas* spp. e.g., *P. fluorescens* and *P. chlororaphis* strains accelerates considerably from refrigerated (around 5 °C) to ambient temperatures and is highest between 25 – 30 °C, after which it rapidly declines. Nevertheless, as it was shown that the proteolytic activity of *P. chlororaphis* and *P. fluorescens* strains culminates at growth temperatures between 15 - 20 °C (Hellio et al., 1993, Nicodeme et al., 2005), temperature must modulate the AprX biosynthesis through an additional mechanism. It was assumed that the regulation of the *aprX-lipA2* operon might be at the post-transcriptional or post-translation level (McKellar and Cholette, 1987, Woods et al., 2001, Dufour et al., 2008), but control at the

transcriptional level also seems possible (McKellar and Cholette, 1987). However, the exact mechanism is not entirely understood yet. Moreover, the lipase LipA2, which is encoded by the gene located at the distal end of the *aprX-lipA2* operon, was shown to underlie low-temperature regulation in members of *P. fluorescens*. Thus, the lipolytic activity decreased continuously with rising incubation temperatures from 16 °C down to a minimum at 31 °C (Woods et al., 2001, Rajmohan et al., 2002). Nevertheless, it is uncertain if temperature directly modulates the LipA2 biosynthesis or if the peptidase AprX, which is co-secreted, possibly degrades LipA2 with elevating temperatures (Duong et al., 1994, Rajmohan et al., 2002).

Furthermore, the AprX biosynthesis of different *P. fluorescens* strains is mediated by iron. Whereas under iron starvation conditions, the *aprX* transcription was assumed to be positively regulated in *P. fluorescens* M114 (Sexton et al., 1995, Sexton et al., 1996, Maunsell et al., 2006), high iron levels caused a repression in *P. fluorescens* B52 (Woods et al., 2001). In *P. aeruginosa* several genes were shown to be negatively regulated under high iron concentration at the transcriptional level by the global regulator Fur (Ferric uptake regulation) (Cornelis et al., 2009), which binds to the specific "Fur-box" upstream of the respective promotor (Vasil and Ochsner, 1999). Whereas Woods et al. observed a repression of *aprX* and *lipA2* transcription in *P. fluorescens* B52 under high iron conditions, Maunsell et al. doubted the influence of Fur on the *aprX* transcription in *P. fluorescens* M114, as the "Fur box" was shown to be located unusually far upstream of the *aprX* promotor (Woods et al., 2001, Maunsell et al., 2006). In contrast, under iron starvation conditions, the alternative σ-factor PbrA was shown to influence the joint production of AprX and the siderophore Pseudobactin (PbuA) in *P. fluorescens* M114. In general, PbrA binds to the "PAD" sequence located in the respective operator region to initiate the transcription of target genes. Moreover, Sexton et al. described a negative regulation of PbrA by Fur (Sexton et al., 1996). However, Maunsell et al. suspected a far more complex regulation of *aprX*, containing an additional PbrA-independent mechanism, probably through siderophores, another σ-factor or modulation directly through iron (Maunsell et al., 2006).

Besides iron, calcium is an essential factor affecting the AprX activity by increasing the stability of the enzyme (Ertan et al., 2015). In several studies, calcium was added to the growth medium, resulting in the elevated proteolytic activity of *P. fluorecens* and *P. chlororapis* strains (Liao and McCallus, 1998, Nicodeme et al., 2005). In addition, other

factors like oxygen or various carbon and nitrogen sources were described to influence the AprX synthesis of Pseudomonas spp., which was reviewed by Zhang et al. and will not be discussed further in the course of this study (Zhang et al., 2019).

4 Detection of milk degradation induced by *Pseudomonas*

Raw milk must contain a total bacterial count of ≤10^5 cfu/ml in the European Union (EU) according to "Act (EG) Nr.853/2004". However, this does not allow a suitable assessment of the raw milk quality, as the enumeration of potential spoilage organisms, such as *Pseudomonas*, must also be taken into account. Therefore, selective cultivation, like agar supplemented with cetrimide, fucidin, and cephalosporin (CFC agar) is used, to enumerate the total *Pseudomonas* cell count (PCC) (figure I 7). Nevertheless, as *Pseudomonas* spp. were shown to be considerably heterogeneous in their proteolytic potential, the determination of PCC is not sufficient to assess the risk of UHT milk decay through heat-stable peptidases (von Neubeck et al., 2015, Baur et al., 2015a, Caldera et al., 2016). Thus, alternative methods exist, detecting the cleavage products originated from casein hydrolysis, determining directly AprX amounts or identifying selected *Pseudomonas* species (Zhang et al., 2019).

In the literature, various colorimetric and fluorimetric methods have been mentioned to detect proteolytic cleavage products of casein. The quantification occurs either directly by detecting the cleavage products of casein in milk or indirectly by isolating the peptidases from milk and utilizing a casein-derivate for the enzyme assay. Among others, azocasein is often applied as casein derivative for the detection of peptidase activity (Baur et al., 2015b, Stoeckel et al., 2016a). The hydrolysis of azocasein leads to the formation of an azo dye, which is colorimetrical quantified. The absorption difference between a blank and the sample represents the apparent enzymatic activity. Thus, the azocasein assay presents an easy to handle, rapid method that, however, lacks sensitivity to determine low peptidase activity. two other spectrophotometric methods are described, namely the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) assay and the o-phthaldialdehyde (OPA) assay (Baur et al., 2015b, Stoeckel et al., 2016a, Machado et al., 2017). In general, both chemicals react with primary amino groups (α-amino groups), which are products of casein hydrolysis, and form a chromophore that is then detected

by a spectrometer. In general, OPA has an advantage over TNBS, as it is more stable, non-toxic, and exhibits a shorter reaction time (Adler-Nissen, 1979, Nielsen et al., 2001). Moreover, the so-called Fluorescamin assay uses a non-fluorescent agent, which forms a fluorescent complex with α-amino groups. While AprX produces small peptides during casein hydrolysis, plasmin creates larger fragments. Thus, to distinguish the hydrolysis products, 12 % TCA (precipitates casein and plasmin cleavage products) and pH 4.6 (precipitates casein) precipitation were performed before the Fluorescamin assay and the subsequent fluorescence measurement. In summary, the Fluorescamin method is a rapid, highly sensitive method with a low detection limit. Nevertheless, it requires more expensive equipment than the TNBS- and OPA-assays, making it less attractive for routinely performed quality diagnostics (Datta and Deeth, 2003).

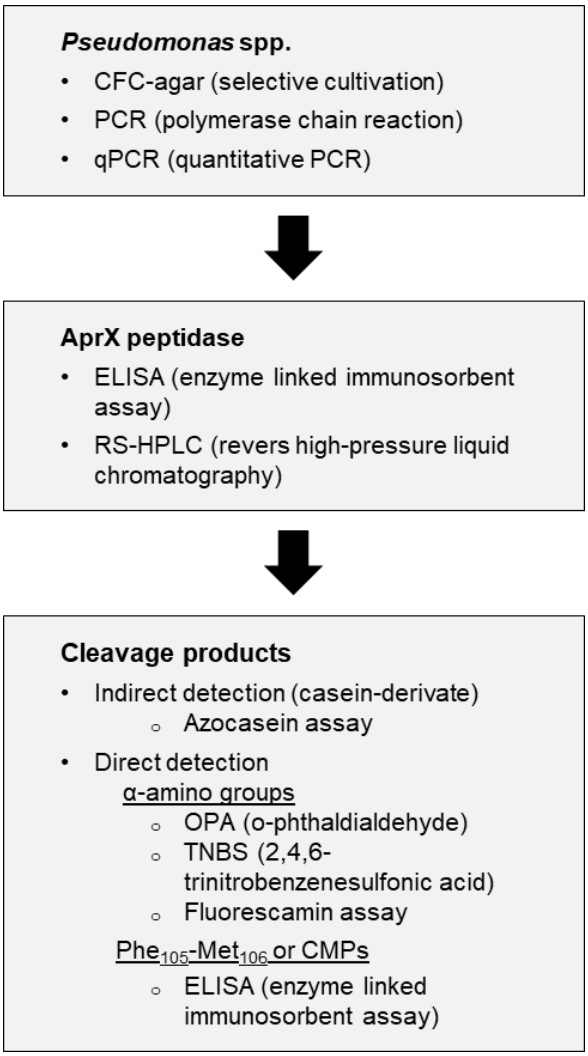

figure I 7: Methods applied to determine proteolytic milk decay. Applications either detect pseudomonads, the AprX peptidase amount or the related cleavage products of casein hydrolysis.

Furthermore, revers high-pressure liquid chromatography (RS-HPLC) was used to analyze the hydrolysis of milk proteins through bacterial peptidases and plasmin in more detail (Datta and Deeth, 2003, Le et al., 2006, Zhang et al., 2018). RS-HPLC is highly sensitive but requires sophisticated equipment and well-trained appliers making it unsuitable for standard diagnostics.

Some studies have also developed various enzyme-linked immunosorbent assays (ELISA) with monoclonal antibodies against different targets to assess UHT milk's spoilage potential. Dupont et al. attempted to detect the entire Phe_{105}-Met_{106} sites in κ-casein, which tend to be hydrolyzed primarily by AprX, Picard et al. aimed to detect the

CMPs formed by caseinolytic digestion (Dupont et al., 2007, Picard et al., 2009). Other studies directly detected the AprX peptidase of different *P. fluorescens* strains or *P. lactis* by ELISA (Birkeland et al., 1985, Clements et al., 1990, Matta et al., 1997, Volk et al., 2021). Although an immunological approach is very promising, the considerable heterogeneity among the AprX structures in different pseudomonads needs to be overcome.

In another approach, individual *Pseudomonas* species were detected by PCR, targeting specifically the *aprX* gene (Martins et al., 2005, Marchand et al., 2009b). However, these assays were either performed in reconstituted skim milk (10 % w/v), which lacks the effect of the milk microbiota and contains less inhibitory substances, possibly reducing the performance of the PCR, compared to whole milk (Marchand et al., 2009b), or in pasteurized milk, where 10^8 cfu/ml pseudomonads were determined as minimal detection limit (Martins et al., 2005). Other studies focused only on members of *P. fluorescens* (Machado et al., 2013), neglecting important milk spoiling species, like *P. proteolytica* or *P. gessardii* (von Neubeck et al., 2015). Besides, the detection of all common pseudomonads by a single primer pair was shown to be difficult, as their *aprX* gene sequences are very heterogeneous (Marchand et al., 2009b).

In conclusion, PCR and ELISA present the most promising approaches to assess the risk of proteolytic milk decay caused by heat-stable peptidases secreted by psychrotolerant *Pseudomonas* species. Nevertheless, both methods suffer from the heterogeneity of either the *aprX* nucleotide sequence or the alterations in the amino acid sequence of AprX within the genus *Pseudomonas.*

5 Overview of applied methods

The methods applied in the three subsections (chapters 1 - 3) of the results are summarized in the following. A more detailed description of the various methods can be found in the respective chapters under "material and methods".

Microbiological methods (chapter 1-3)

- Standard cultivation of bacteria on solid and in liquid medium. For molecular cloning or conjugation, the medium was supplemented with the required antibiotics or sucrose

- Selective cultivation on CFC-agar for the enumeration of *Pseudomonas* cell counts (PCC) by plating
- Cultivation of *P. proteolytica* WS 5128 in UHT milk (1.5 % fat) to analyze various regulatory levels of the AprX biosynthesis and to determine the proteolytic potential of various *Pseudomonas* species

Enzymatic assays (chapter 1, 3)

- Cultivation of different *Pseudomonas* spp. on skim milk agar to determine the total proteolytic activity by measurement of the clearing zones (agar diffusion assay)
- Quantification of the extracellular proteolytic activity via azocasein assay

Whole-genome sequencing (WGS) of *Pseudomonas* spp. (chapter 1)

- Isolation of genomic DNA and library preparation for Illumina sequencing
- Whole-genome sequencing using Illumina MiSeq System

Establishment of the multiplex qPCR assay (chapter 2)

- Design of primers and hydrolysis probes for specific binding to target DNA sequences and determination of their amplification efficiency
- Evaluation of the qPCR assay's sensitivity and specificity
- Quantification of the total *Pseudomonas* cell counts to compare selective plating and the universal *Pseudomonas* probe included in the multiplex qPCR assay

Molecular cloning (chapter 3)

- Construction of recombinant plasmids (applying, specific primer design, PCR, agarose-gel-electrophoresis, restriction digest, ligation, dephosphorylation, plasmid isolation, and plasmid) and electro-transformation into competent *E. coli* cells
- Conjugational plasmid transfer from *E. coli* (donor) to *P. proteolytica* (acceptor)
- Production of *P. proteolytica* deletion mutants through homologs recombination and verification by PCR and gene sequencing

Transcription analysis (chapter 3)

- RNA extraction from cell pellets and reverse transcriptase PCR
- SYBR-Green qPCR using cDNA as template
- Relative expression analysis

Statistics (chapter 1-3) were used to determine the significance of received data from bacterial growth in milk, transcription analysis, and azocasein-assay

6 The aim of the project

Psychrotolerant *Pseudomonas* spp. dominate the microbiota of refrigerated raw milk and secrete heat-stable peptidases, reducing the quality of UHT milk and UHT milk products. However, the amount of peptidases produced is difficult to predict, as it relies on genetic factors, which are species- and partly strain-specific and is influenced through various environmental parameters. Because the removal of the peptidases from the end product is challenging and the inactivation is nearly impossible, the proteolytic potential of the individual pseudomonads must be determined, and influences favoring high enzyme production elucidated. Thus, this study aimed to identify potential milk spoiling *Pseudomonas* spp. and analyze the effect of genetic and environmental factors on the AprX biosynthesis. Moreover, a detection system should be established to quantify the most common *Pseudomonas* species in raw milk. For these purposes, the study was divided into three chapters (figure I 8).

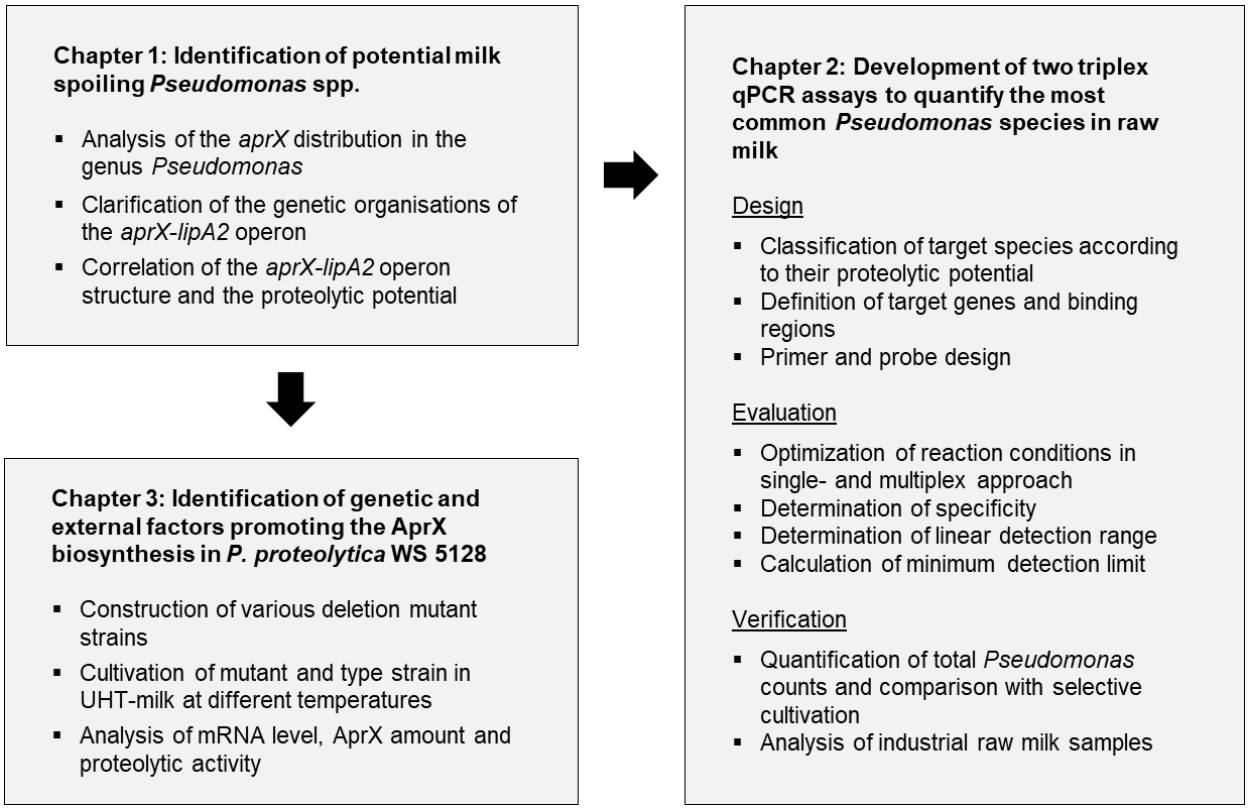

figure I 8: Overview of the study, which was divided into three parts (chapter 1 -3). The relationships of the different chapters are indicated.

The *aprX* locus presents the basic requirement for proteolytic activity of milk-associated *Pseudomonas* species. However, the distribution of *aprX* in the genus *Pseudomonas* was not analyzed yet. To close this gap, the presence of *aprX* among 185 *Pseudomonas*

type strains was examined. Moreover, the genetic organization of the *aprX-lipA2* operon was analyzed, and the correlation with the proteolytic potential of 129 *Pseudomonas* strains, mainly isolated from milk, was investigated. Received findings were fundamental for chapters 2 and 3.

One way to reduce heat-stable peptidases in raw milk is to avoid conditions, advancing their production. Thus, genetic and environmental factors on the AprX biosynthesis of *P. proteolytica* WS 5128 were examined in chapter 2. Therefore, the wild type and constructed deletion mutants (*P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*) were cultivated in milk at 12 °C and 30 °C. Subsequently, the transcription levels of 5 operon genes (*aprX*, *aprD*, *prtA*, *prtB* and *lipA2*), the secreted AprX amount, and the extracellular proteolytic activity were determined at specific sampling points. These findings contribute to the elucidation of the complex regulatory mechanisms modulating the AprX biosynthesis.

Pseudomonas species vary enormously in their proteolytic activity and their abundance in milk, which is why the determination of the total *Pseudomonas* counts to assess raw milk quality is not sufficient. Thus, in chapter 3, two triplex qPCR assays to quantify the most common *Pseudomonas* species in raw milk were established. For this purpose, five species-specific hydrolysis probes and one to detect all members of *Pseudomonas* were developed. Moreover, amplification efficiency, sensitivity, and specificity of all hydrolysis probes were determined. Furthermore, the total *Pseudomonas* counts were determined with the universal *Pseudomonas* hydrolysis probe and the results were compared with selective plating. Besides, the composition of the *Pseudomonas* population in 60 raw milk samples was successfully analyzed, revealing the usefulness of the developed method to detect the most common *Pseudomonas* species in raw milk according to their proteolytic activity.

II RESULTS

1 Chapter overview

Chapters 1 and 2 of the result section were published previously, and the original publications are added. Chapter 3 is presented as a drafted manuscript. Personal contributions of the authors are mentioned.

Chapter 1

Maier, C., Huptas, C., von Neubeck, M., Scherer, S., Wenning, M., Lücking, G. (2020). Genetic organization of the *aprX-lipA2* operon affects the proteolytic potential of *Pseudomonas* species in milk. Front Microbiol. 11, 1190. doi: 10.3389/fmicb.2020.01190

Personal Contribution: CM: library preparation, genome sequencing, agar diffusion assays, azocasein assays, data analysis and visualization, manuscript drafting, and writing. CH: library preparation, genome sequencing, phylogenomic analysis, bioinformatics analysis, statistical analysis, data analysis and visualization, manuscript drafting, and writing. MN: library preparation, genome sequencing, and data analysis. SS: data discussion, manuscript revision. MW: data discussion, manuscript revision, project planning. GL: data discussion, manuscript revision, project supervision. All authors read and approved the final manuscript.

Chapter 2

Maier, C., Hofmann, K., Huptas, C., Scherer, S., Wenning, M., Lücking, G. (2021) Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR. Appl Microbiol Biotechnol 105 (4), 1693-1708. doi: 10.1007/s00253-021-11109-0

Personal Contribution: CM: performed research, analyzed data, and wrote manuscript; KH: conducted experiments and analyzed data; CH: prepared phylogenetic trees and wrote sections of material and methods; SS: discussed and revised manuscript; MW: designed study and revised manuscript; GL: planned study and wrote manuscript. All authors read and approved the manuscript.

Chapter 3

Maier, C, Volk, V, Wenning, M, Fischer, L, Scherer S, Lücking, G

Regulatory effects of *aprX-lipA2* operon genes, temperature and growth phase on AprX production in *Pseudomonas proteolytica* WS5128*, unpublished*

Personal Contribution: CM: conducted cloning, construction of deletion mutants, azocasein assay, RNA extraction, reverse transcription, and qPCR analysis, data analysis, data visualization, wrote the first draft; VV and LF: provided anti-AprX specific antibodies and conducted western blot analysis; SS and MW: data discussion and design of the study; GL: planned study and edited manuscript.

2 Chapter 1: "Genetic organization of the *aprX-lipA2* operon affects the proteolytic potential of *Pseudomonas* species in milk."

Summary

Every year, premature spoilage of ultra-high temperature (UHT) treated milk results in economic losses for the milk industry. One reason for proteolytic milk decay is psychrotolerant *Pseudomonas* species secreting the heat-stable metallopeptidase AprX, which is encoded by the first gene of the *aprX-lipA2* operon. While the proteolytic potential of many pseudomonads was previously characterized, very little is known about the *aprX* distribution among the genus *Pseudomonas* and the genetic organization of the *aprX-lipA2* operon.

In this study, the *aprX* locus was detected in 81 out of 185 different *Pseudomonas* type strains through a phylogenomic approach, whereby most of these species were assigned to a monophyletic group. Moreover, the majority of milk-isolated pseudomonads were allocated either to the *P. gessardii*, *P. fluorescens,* or *P. fragi* subgroup. In total, 22 distinct *aprX-lipA2* operon types were identified, of which almost all could be separated into a conserved region containing five core genes (*aprXIDEF*) and a variable part including two putative autotransporter genes (*prtA* and *prtB*) and up to two lipases (*lipA1* and *lipA2*). The most frequently found operon structure was type 1 (*aprXIDEF prtAB lipA2*), which was present in 31 % of all analyzed strains harboring *aprX*. Other operon structures mainly varied in the arrangement, existence, and localization of genes included in the variable operon part. Furthermore, the proteolytic activity of 129 different *Pseudomonas* strains was semi-quantitatively determined on skim milk agar and could be associated in parts with the genetic organization of the operon structure. For representatives with operon type 1 and 9 (*aprXIDEF prtAB lipA2* | *lipA1*), elevated proteolytic activities were determined compared to strains comprising other structures. Thereby, the localization of*prtA* and *prtB* within the *aprX-lipA2* operon correlated significantly with increased caseinolytic potential.

In summary, the distinct genetic organizations of the *aprX-lipA2* operon displayed the high genetic variability of *Pseudomonas* spp. and indicated a correlation of the operon structure with proteolytic activity. Moreover, our findings highlight the species- and strain-dependent proteolytic potential in the genus *Pseudomonas* and indicate a highly complex AprX biosynthesis including several intrinsic and extrinsic factors.

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Genetic Organization of the *aprX-lipA2* Operon Affects the Proteolytic Potential of *Pseudomonas* Species in Milk

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Psychrotolerant *Pseudomonas* species are a main cause of proteolytic spoilage of ultra-high temperature (UHT) milk products due to the secretion of the heat-resistant metallopeptidase AprX, which is encoded by the first gene of the *aprX-lipA2* operon. While the proteolytic property has been characterized for many different *Pseudomonas* isolates, the underlying *aprX-lipA2* gene organization was only described for a few strains so far. In this study, the phylogenomic analysis of 185 *Pseudomonas* type strains revealed that the presence of *aprX* is strongly associated to a monophylum composed of 81 species, of which 83% carried the *aprX* locus. Furthermore, almost all type strains of known milk-relevant species were shown to be members of the three monophyletic groups *P. fluorescens*, *P. gessardii*, and *P. fragi*. In total, 22 different types of *aprX-lipA2* genetic organizations were identified in the genus, whereby 31% of the species tested carried the type 1 operon structure consisting of eight genes (*aprXIDEF prtAB lipA2*). Other genetic structures differed from type 1 mainly in the presence and location of genes coding for two lipases (*lipA1* and *lipA2*) and putative autotransporters (*prtA* and *prtB*). The peptidase activity of 129 strains, as determined on skim milk agar and in UHT-milk, correlated largely with different *aprX-lipA2* gene compositions. Particularly, isolates harboring the type 1 operon were highly proteolytic, while strains with other operon types, especially ones lacking *prtA* and *prtB*, exhibited significantly lower peptidase activities. In conclusion, the phylogenomic position and the *aprX-lipA2* gene organization specify the proteolytic potential of *Pseudomonas* isolates. In addition, however, an interplay of several environmental factors and intrinsic traits influences production and activity of AprX, leading to strain-specific proteolytic phenotypes.

Keywords: *aprX-lipA2* operon, AprX peptidase, genus *Pseudomonas*, proteolytic potential, milk spoilage

INTRODUCTION

Psychrotolerant bacteria, predominant in cold stored raw milk, are known to secrete heat-resistant enzymes, which partly even withstand ultra-high temperature processing (von Neubeck et al., 2015; Glück et al., 2016). Continuous enzyme activity during storage can cause product defects like off-flavors and textural flaws by decomposition of milk components, primary casein (Stoeckel et al., 2016). As a consequence, the quality of ultra-high temperature treated (UHT) products decreases and premature spoilage may occur, presenting considerable economic challenges for the milk industry (McKellar, 1981; Sørhaug and Stepaniak, 1997; Stoeckel et al., 2016). Especially, UHT products intended for long-distance transport, e.g., export to Asia, require prolonged shelf life to ensure consistent product stability and quality.

Representatives of the genera *Pseudomonas*, *Microbacterium*, *Acinetobacter*, and *Lactococcus* are particularly abundant in cold stored raw milk. Among them, pseudomonads were shown to be the main cause of proteolytic activity (Baur et al., 2015a; von Neubeck et al., 2015). Currently, the genus *Pseudomonas* comprises 188 species (March 2019), which makes it one of the largest bacterial genera known so far. *Pseudomonas* species are ubiquitous, gram-stain negative, non-spore forming, aerobic rods (Palleroni, 2015). After frequent reclassifications, the genus is presently divided into 21 monophyletic groups (Gomila et al., 2015; Peix et al., 2018). With respect to spoilage potential, previous studies revealed that milk-associated *Pseudomonas* strains differ strongly in their proteolytic properties, even if they are taxonomically closely related (Baur et al., 2015a). Apart from the cultivation temperature, other extrinsic and intrinsic factors such as the composition of the cultivation medium, iron content, quorum sensing, and phase variation were shown to influence peptidase activities of *Pseudomonas* members in a species- and partially strain-specific manner (Nicodeme et al., 2005; Maunsell et al., 2006; Liu et al., 2007; Alves et al., 2018). However, the complex regulatory processes behind these variations are not yet fully understood.

So far, only one extracellular peptidase has been characterized in *Pseudomonas*, namely the metallopeptidase AprX (Liao and McCallus, 1998; Woods et al., 2001). The nomenclature of AprX in literature is partly misleading as AprX was also entitled AprA in *P. aeruginosa* (Guzzo et al., 1991). However, in the context of proteolytic, milk-associated species, AprA and AprX are identical, whereas in *P. aeruginosa aprX* and *aprA* encode for two different proteins (Duong et al., 2001). The caseinolytic endopeptidase AprX belongs to the serralysin family and holds a Zn^{2+} ion and Ca^{2+} ions for stability and functionality (Schokker and van Boekel, 1997). It has a size of 45–50 kDa (Marchand et al., 2009), shows the highest substrate turnover at 37–45°C and is functional from slightly acidic to alkaline pH (Dufour et al., 2008; Marchand et al., 2009; Martins et al., 2015; Matéos et al., 2015). The corresponding *aprX* gene is located in the *aprX-lipA2* operon, consisting of several genes controlled by a single promotor upstream of the *aprX* gene. In *P. fluorescens* B52, the operon includes, besides *aprX*, genes encoding for a peptidase inhibitor (AprI), a type I secretion system (AprDEF), two putative autotransporter homologs (PrtAB), and a lipase (LipA2) (Woods et al., 2001). So far, the *aprX-lipA2* gene cluster is known to comprise up to nine different genes in total (Ma et al., 2003). Strain-specific deviations in the organization of the operon, for example, a missing *prtA* gene in *P. brassicacearum* NFM421, loss of *prtAB* in *P. fluorescens* SIK W1, an additional lipase gene in *P. fluorescens* Pf0-1, and a completely different operon structure in *P. aeruginosa* PAO-1 have been mentioned in the literature (Duong et al., 2001; Ma et al., 2003). However, the *aprX-lipA2* operon structure has only been described for very few *Pseudomonas* species up to now.

To close this gap, the aims of this study were an extensive analysis of the *aprX* gene distribution in the whole genus *Pseudomonas* as well as the clarification of the existing *aprX-lipA2* genetic organizations and their possible influence on peptidase production. Toward this end, a phylogenomic analysis of 185 type strains based on 92 bacterial core genes was conducted and the strain set screened for *aprX*-positive candidates. Besides, 87 *Pseudomonas* isolates, mainly from raw milk, were fully sequenced and the *aprX-lipA2* operon organization of all strains analyzed. Moreover, the proteolytic activity of selected strains with different genetic constitutions was determined in order to find potential correlations between peptidase production and the underlying operon structure.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Pseudomonas strains used for sequencing and/or proteolytic profiling (**Supplementary Tables S1** and **S2**) were grown aerobically on tryptic soy agar (TSA, Carl Roth GmbH) for 24 h at 30°C. For overnight cultures, 4 ml tryptic soy broth (TSB, Merck Millipore KGaA) were inoculated with material from one colony and incubated for 16 h at 30°C and 150 rpm.

DNA Extraction

Genomic DNA (gDNA) was extracted from overnight cultures using the QIAamp® DNA Mini Kit (Qiagen). In contrast to the manufacturer's instructions, samples were treated with Proteinase K for 4 h at 56°C. RNA digestion was performed for 30 min at 70°C using 10 µl of RNase A at a concentration of 10 mg/ml (Thermo Scientific). Final elution of gDNA from spin columns was carried out twice with 100 µl of sterile deionized water each. DNA concentrations were measured using a Qubit 2.0 Fluorometer (Invitrogen) and Qubit dsDNA HS Assay Kits (Invitrogen) in compliance with the manufacturer's instructions.

Whole-Genome Sequencing, Read Quality Control, and *de novo* Assembly

All 87 *Pseudomonas* strains sequenced *de novo* are summarized in **Supplementary Table S1**. For each strain, at least one sequencing library was prepared according to a modified version of the Illumina TruSeq DNA PCR-free Sample Preparation

procedure (Huptas et al., 2016). Libraries were sequenced with the Illumina MiSeq System. Almost all sequencing runs were conducted using MiSeq Reagent v3 Kits (600-cycle) or v2 Kits (500-cycle). Library pools were demultiplexed with the on-board MiSeq Reporter Software. After visual inspection of raw read quality using FastQC v0.10.1[1], reads were trimmed and filtered with the NGS QC Toolkit v2.2.3 (Patel and Jain, 2012). Reads were cut 10 nts from 5′ end and at least 1 nt from 3′ end. Low quality and adapter contaminated reads as well as those losing their counterpart during filtering were rejected. Finally, repeated visual analysis (FastQC) ensured that all remaining reads were of high quality. **Supplementary Table S1** shows the read lengths and sequencing depths achieved for each individual sample. Genome reconstruction was performed with SPAdes v2.5.1 (Bankevich et al., 2012) using the assembler's in-build functionalities for read error (Nikolenko et al., 2013) and contig mismatch correction. In any case, k-mers applied were 21, 33, 55, 77, 99, and 127. Contigs with less than 500 nts were removed from draft genome assemblies. For each sequenced strain, detailed assembly statistics are listed in **Supplementary Table S1**.

Public Genome Data

Next to the 87 strains sequenced and assembled *de novo* (**Supplementary Table S1**), 184 *Pseudomonas* genome assemblies (**Supplementary Table S3**) were obtained from the National Center for Biotechnology Information (NCBI).

Phylogenomic Tree Reconstruction and Monophyletic Group Assignment

The UBCG pipeline v3.0 (Na et al., 2018) was applied to extract and align 92 universal bacterial core genes from genome sequence data. In general, standard parameter settings were applied with the exception of the filter cutoff that was set to zero. On the basis of the UBCG multiple sequence alignments, maximum likelihood phylogenomic trees were calculated using the MEGA X Software v10.0.5 (Kumar et al., 2018). To model DNA evolution, the General-Time-Reversible model incorporating rate heterogeneity (five discrete gamma categories) and a proportion of invariant sites (GTR+G+I) was chosen. Positions containing gaps in the multiple sequence alignments were not considered. All remaining parameters were kept at their default settings. In total, 200 bootstrap replicates were computed for each tree to infer branch confidence values. Bacterial strains present in both phylogenomies (**Figures 1, 2**) were separated into distinct clades according to a classification scheme already used to highlight the intrageneric structure of the genus *Pseudomonas* based on multi-gene phylogenies (Mulet et al., 2010; Gomila et al., 2015; Peix et al., 2018). In more detail, the positioning of each strain within the phylogenomy was used to assign it to one of the 21 monophyletic groups defined previously. In cases in which topological constrains (length and order of branches) prohibited the allocation to a known group, strains were kept as singletons (no group membership) or clustered together to form a new group. New groups were named after the first species described in that group. Both phylogenomies were visualized using the Interactive Tree Of Life (iTOL) online tool v5.3 (Letunic and Bork, 2019).

[1]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Species Delineation and Naming of Strains

Species affiliation of non-type strains was determined using the Microbial Species Identifier (MiSi) software v1 (Varghese et al., 2015). First, each non-type strain was compared to all type strains and assigned to the particular nomenspecies, to which it shared the highest genome-wide average nucleotide identity (gANI), provided the observed gANI value was not less than the threshold for species demarcation (96.5%). Non-type strains not assignable in that way ($max(gANI) < 96.5\%$) were analyzed in a subsequent all-against-all comparison. Finally, non-type strains sharing gANI values above the species cutoff were clustered together. Based on this analysis, our strain collection contains 12 potentially new genomospecies. Non-type strains were named in accordance to the species they were assigned to during species delineation.

Gene Prediction and Screening of *aprX-lipA2* Operon Genes

Prodigal v2.6 (Hyatt et al., 2010) was applied to predict the protein coding potential of each strain (**Supplementary Tables S1** and **S3**) preventing genes to run off contig ends and forcing Shine-Dalgarno motif scans. Predicted gene sequences as well as corresponding protein translations were used for further analysis. A reference protein sequence of each of the nine known *aprX-lipA2* operon genes (GenBank IDs AGL85002.1 to AGL85010.1) was used to search for homologous sequences within the predicted protein coding content of each strain/genome investigated. For each strain and *aprX-lipA2* reference protein the best BLASTp v2.2.25+ (Camacho et al., 2009) hit was saved. Subsequently, best hits were part of manual curation taking alignment statistics (bit-score, e-value, percent identity, aligned fraction, etc.) and gene neighborhood into account to judge whether a best hit corresponds to an orthologous protein. If necessary, genomic loci of best hits were analyzed in detail to uncover single-nucleotide polymorphism and small insertion/deletion mutations. Multiple sequence alignments of genomic loci and gene sequences were calculated online using Clustal Omega v1.2.4 (Sievers and Higgins, 2014).

Statistics

All statistical analyses were performed with R v3.6.1 (R Core Team, 2019). To test for nonrandom associations Fisher's exact test (Fisher. test function of stats package) was used. One-way analysis of variance (aov function of stats package) in combination with Tukey's multiple comparison test [Tukey_hsd function of rstatix package (Kassambara, 2019b)] was applied to test for significant differences in mean proteolytic activity (agar diffusion assay at days 3, 4, and 7) at species level of the most abundant *aprX-lipA2* genetic organizations (**Figure 4** and **Supplementary Figure S1**). Associated boxplot

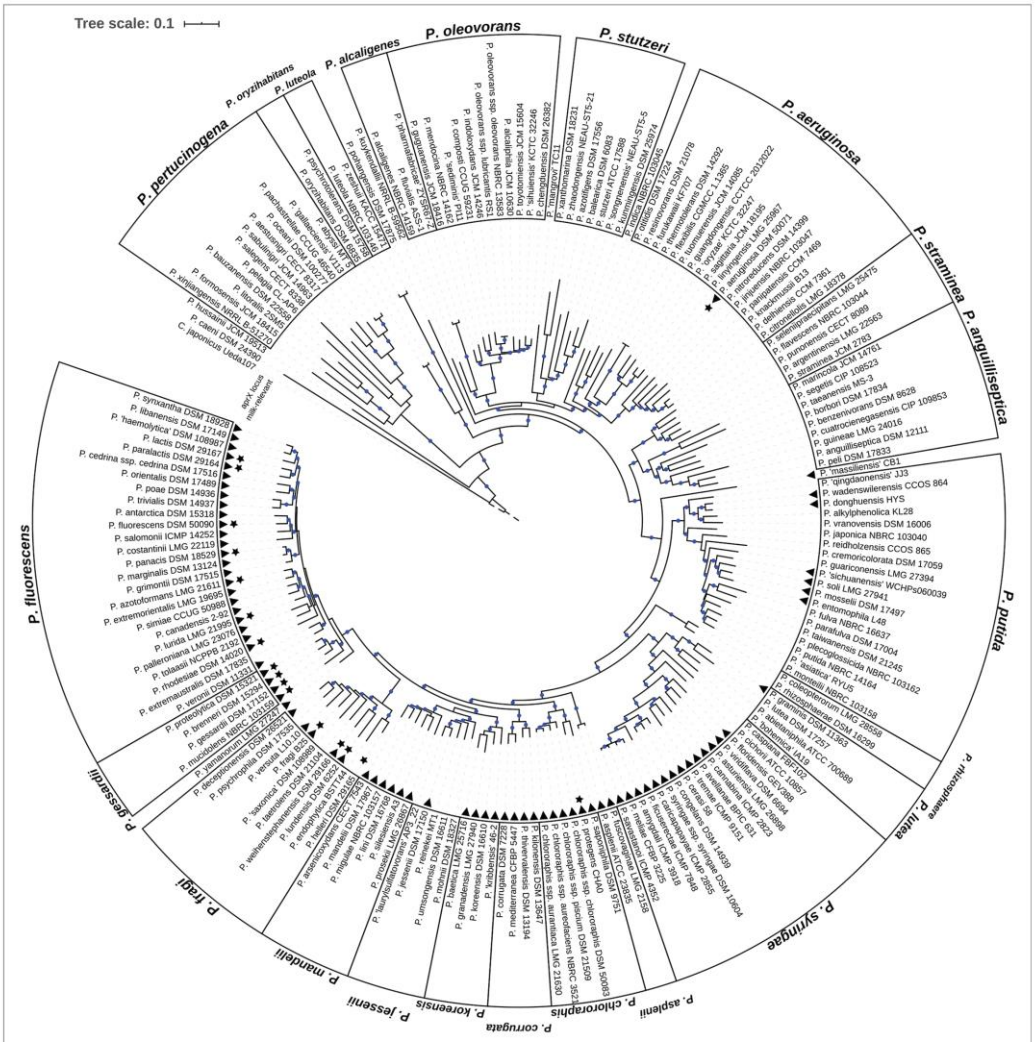

FIGURE 1 | Phylogenomic tree of the genus *Pseudomonas*. Shown is the rooted maximum likelihood phylogenomy of the genus *Pseudomonas* comprising 185 type strains. The tree is based on a multiple sequence alignment composed of 92 universal bacterial core genes (69,704 alignment positions). Evolutionary distances were estimated using the GTR+G+I model. Branches with high bootstrap support (≥70%) are marked with blue circles. In total, 200 bootstrap replicates were calculated. *Cellvibrio japonicus* Ueda107^{T} (RefSeq ID NC_010995.1) is used as outgroup. For reasons of clarity the length of the outgroup branch was downscaled to 0.05 substitutions per site (dashed branch). Black triangles highlight type strains containing the *aprX* gene. Black stars refer to type strains of species classified as milk-relevant in the past. The genus is separated into 22 distinct monophyletic groups and 8 singletons. Group names are shown at the outer-most border.

representations were created using functionalities of the R packages ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2019a), ggthemes (Arnold, 2019), cowplot (Wilke, 2019), grid, and gridExtra (Auguie, 2017). To meet test assumptions, normality of data and homogeneity of variance were checked with the Shapiro-Wilk (Shapiro.test function of stats package) and Levene's test [LeveneTest function of car package (Fox and Weisberg, 2019)], respectively (**Supplementary Table S4**). In general, strains showing no proteolytic activity until day 7 (zero values) were omitted from statistical analyses to avoid a distortion of the test statistics.

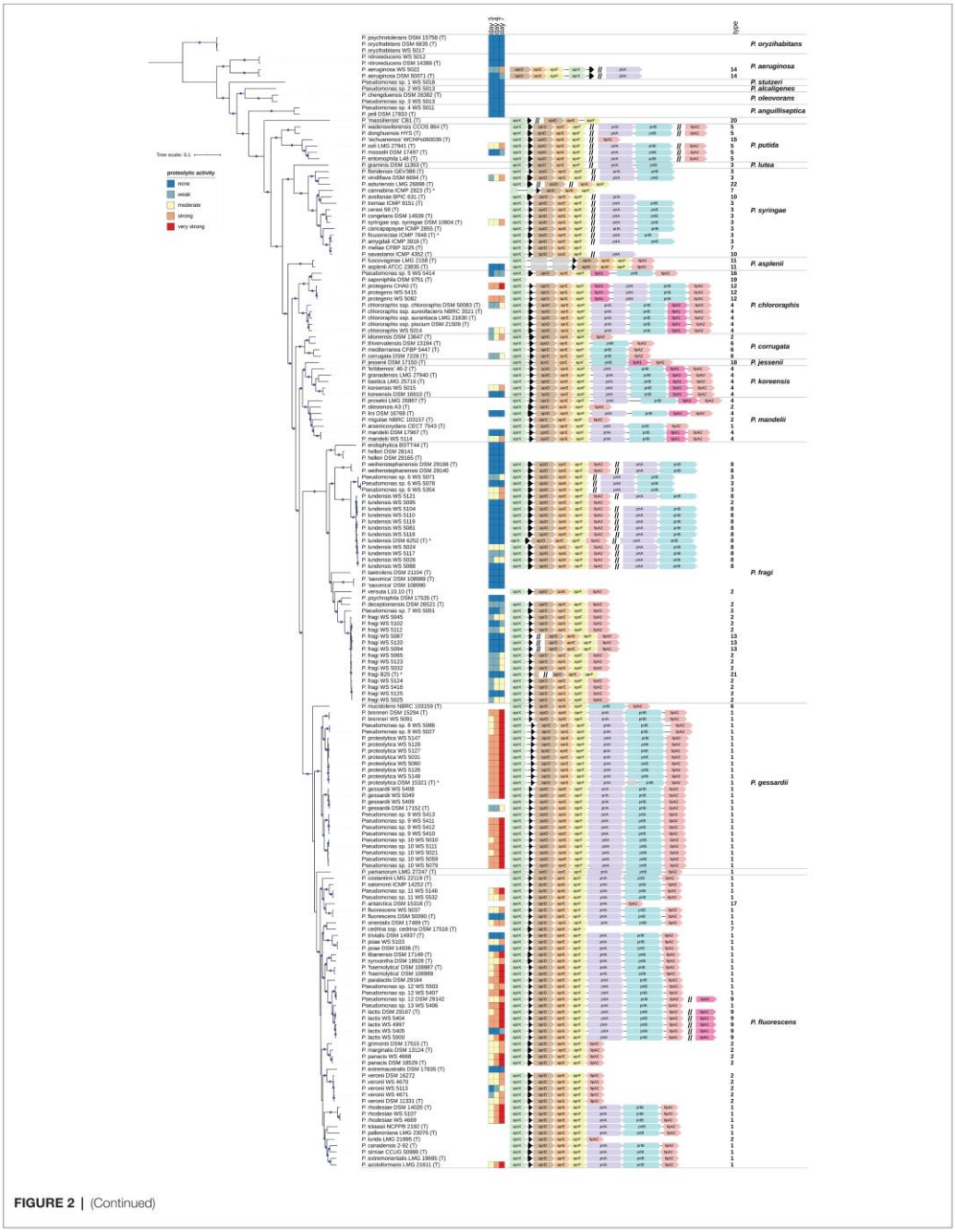

FIGURE 2 | (Continued)

FIGURE 2 | Proteolytic activity and *aprX-lipA2* genetic organizations of selected *Pseudomonas* strains. The rooted phylogenomy shows 178 *Pseudomonas* strains for which an agar diffusion assay was performed and/or the *aprX* gene was found. The maximum-likelihood method and the GTR+G+I model were applied to reconstruct the tree based on 92 concatenated universal bacterial core genes (75,078 alignment positions). Branches containing blue circles are of high bootstrap support (≥70% of 200 replicates). The monophylum represented by strains *P. psychrotolerans* DSM 15758^T, *P. oryzihabitans* DSM 6835^T, and *P. oryzihabitans* WS 5017 was used to root the tree (outgroup). Strain-specific proteolytic activity after 3, 4, and 7 days is visualized as heatmap illustrating non (dark blue), weak (light blue), moderate (yellow), strong (light red) and very strong (dark red) activities. *AprX-lipA2* genetic organizations are shown at single-nucleotide resolution next to the heatmap and in a strain-wise manner. Coding sequences not belonging to one of the genes *aprX* (green), *aprI* (black), *aprD* (brown), *aprE* (orange), *aprF* (yellow), *prtA* (magenta), *prtB* (cyan), *lipA1* (pink), and *lipA2* (antique pink) are colored gray. Individual genes or gene clusters of the *aprX-lipA2* operon being present at distinct genomic locations are separated by a double slash (//). In such cases, the different portions of genetic information do not necessarily have to lie on the same strand (be encoded in the same direction). Strains containing partial gene sequences are marked with an asterisk (*). In this context, left-open gene sequences indicate that the 5'-portion of the coding sequence is missing, whereas right-open gene sequences highlight cases lacking the 3'-part. Each *aprX-lipA2* genetic organization is followed by its type number. To provide a more structured overview the phylogenomy is divided into 18 monophyletic groups and two singletons (*P. massiliensis* CB1^T and *P. yamanorum* LMG 27247^T). Group names are listed on the right-hand of the figure.

FIGURE 3 | Most abundant *aprX-lipA2* genetic organizations. Schematic representation of the four most abundant *aprX-lipA2* genetic organizations (type 1–4) in *Pseudomonas*, as determined by number of species per type. The double slash (//) indicates the presence of particular gene clusters at distinct genomic locations.

Measurement of Proteolytic Activity

Supplementary Table S2 lists 129 *Pseudomonas* strains for which agar diffusion assays or azocasein assays were performed. The strain set comprises 72 isolates from raw milk or semi-finished milk products, four isolates from water, two from food environment, one from a cow, one from soil, and three with unknown origin [Weihenstephan (WS) strain collection, TUM, Freising; DSMZ, Braunschweig], and 46 *Pseudomonas* type strains of diverse origins including raw milk (DSMZ, Braunschweig). For the agar diffusion assay, proteolytic activity of *Pseudomonas* strains was determined on skim milk agar [2× nutrient agar with 10% (w/v) skim milk powder; pH 6.8]. After suspending one loop of cell material in 100 µl sterile deionized water, the suspension was firmly vortexed and 15 µl were spotted on skim milk agar. After cultivation for 7 days at 12°C, clearing zones around the colonies indicated the proteolytic hydrolysis of casein. The extent of enzymatic activity Δr was assessed by subtracting the radius of the colony r_{colony} from the radius of the clearing zone r_{zone} in cm ($\Delta r = r_{zone} - r_{colony}$). Thereby all tested strains were categorized into non (Δr = 0 cm), weak (0 cm < Δr ≤ 0.2 cm), moderate (0.2 cm < Δr ≤ 0.5 cm), strong (0.5 cm < Δr < 1 cm), and very strong Δr ≥ 1 cm) peptidase producers. The azocasein assay was used to quantify the extracellular proteolytic activity in liquid cultures (Sørhaug and Stepaniak, 1997). Therefore, an overnight culture was used to inoculate 50 ml UHT milk (1.5% fat) with approximately 10^3–10^4 cfu and the culture was shaken at 150 rpm at 12°C for 4 days. Bacterial cultures were harvested (14,000 rpm, 12°C), the supernatants carefully sterile-filtered (0.22 µm; Berrytec GmbH) and frozen at −20°C until use. In parallel, cell counts were determined for each sample on TSA. Before measurement, the supernatants were diluted 10^{-1} and 10^{-2} in Ringer solution (Merck KGaA) and the azocasein assay was performed as previously described (Baur et al., 2015b) with slight modifications. In brief, the diluted supernatants and a freshly prepared azocasein-solution containing 0.5% (w/v) azocasein (Sigma-Aldrich), 50 mM MOPS buffer (pH 6.7), and 1 mM $CaCl_2$ were preincubated separately at 40°C for 5 min. Then, 100 µl azocasein-solution was added to 100 µl of diluted supernatant and samples were incubated at 40°C for 1 h while shaking (600 rpm). The enzymatic reaction was stopped by adding 20 µl 2 M TCA to the sample. After centrifugation (14,000 rpm, 5 min), 150 µl of each sample was transferred into a microtiter plate containing 50 µl 1 M NaOH. Absorbance was measured at 450 nm in a plate reader (Victor3, PerkinElmer Inc.) and only absorption values below 0.59 were stated as linear and taken into account. Proteolytic activity (Sørhaug and Stepaniak, 1997) was defined as difference between absorption of blank and sample value at 450 nm per hour and ml enzyme solution ($\text{EPA} = \frac{\Delta A}{\text{h x ml}}$). EPA values were normalized to the logarithm of 10^9 cells and the according strains were categorized into low (0 ΔA/h ml ≤ EPA ≤ 250 ΔA/h ml), middle (250 ΔA/h ml < EPA ≤ 500 ΔA/h ml) and high (EPA > 500 ΔA/h ml) peptidase producers.

RESULTS

Pseudomonas Phylogenomy

Evolutionary relationships between the species of the genus *Pseudomonas* were estimated by a phylogenomic treeing approach. As of March 2019 the List of Prokaryotic names with Standing in Nomenclature (LPSN; Parte, 2014) contained 188 *Pseudomonas* species with validly described names, of which *P. brassicacearum*, *P. chlororaphis*, *P. oleovorans*, and *P. syringae* can be further divided into nine subspecies (**Supplementary Table S5**). A total of 185 type strains representing 87.8% (165) of the *Pseudomonas* species with validly published names were used and a maximum-likelihood (ML) phylogenomic tree was calculated on the basis of 92 universal bacterial core genes (**Figure 1**).

Based on the topology of the phylogenomy, 175 type strains were assigned to 21 monophyletic groups suggested previously by means of multi-locus-sequence-analysis (MLSA) (Mulet et al., 2010; Gomila et al., 2015; Peix et al., 2018). In addition, a new group (*P. alcaligenes*) consisting of the type strains

P. alcaligenes NBRC 14159^T, *P. fluvialis* ASS-1^T and *P. pharmafabricae* ZYSR67-Z^T was formed. With few exceptions, group memberships remained unchanged compared to the above mentioned MLSA analysis. Only two type strains changed their position and were moved from the *P. aeruginosa* monophylum to the *P. anguilliseptica* (*P. cuatrocienegasensis* CIP 109853^T) and *P. alcaligenes* (*P. alcaligenes* NBRC 14159^T) group. Furthermore, two type strains without former group affiliation (*P. aestusnigri* CECT 8317^T and *P. salegens* CECT 8338^T) were placed in the *P. pertucinogena* group. In total, 23 of the type strains investigated were not part of previous MLSA phylogenetic studies. **Supplementary Table S6** lists the phylogenomic group memberships of these strains.

Distribution of the *aprX* Gene Within the Genus

To shed light on the distribution of the *aprX* gene within the genus, protein-coding sequences of the 185 type strains were screened for the peptidase AprX using BLASTp (Camacho et al., 2009). Homologs were found in 81 type strains (black triangles in **Figure 1**). In five of these strains, genome assemblies indicate that the *aprX* genes might not be functional due to the occurrence of mutations (**Supplementary Table S7**). Hence, the overall frequency of *aprX* carrying type strains in the genus corresponds to 43.8%. However, type strains harboring *aprX* are not evenly distributed across the genus. The presence of *aprX* is strongly associated to the monophylum composed of the phylogenetic groups *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata*, *P. chlororaphis*, *P. asplenii*, *P. syringae*, and *P. lutea* (Fisher's exact test, $p < 2.2 \times 10^{-16}$, odds-ratio ~ 52.1). Indeed, 83% (73 of 88) of the monophylum's type strains carry *aprX*, whereas the gene is only present within 8.2% (8 of 97) of the remaining type strains (**Figure 1**). Apart from few exceptions (*P. fragi* and *P. putida*), *aprX* is present (≥80%) or absent (≤20%) in almost all members of the different monophyletic groups (**Supplementary Table S8**).

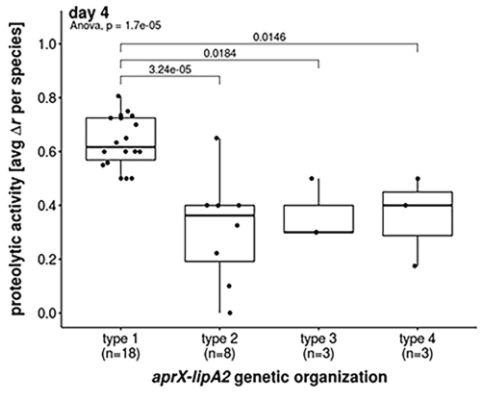

FIGURE 4 | Proteolytic activities of most abundant *aprX-lipA2* genetic organizations. Boxplot representation of proteolytic activities at day 4 with regard to the most abundant *aprX-lipA2* genetic organizations, namely *aprXIDEF prtAB lipA2* (type 1), *aprXIDEF lipA2* (type 2), *aprXIDEF | prtAB* (type 3) and *aprXIDEF prtAB lipA1A2* (type 4). Each dot refers to the average proteolytic activity of a particular *Pseudomonas* species. One-way ANOVA revealed a significant difference in mean proteolytic activity between *aprX-lipA2* genetic organizations ($p = 1.7 \times 10^{-5}$). *Post hoc* Tukey's Test indicates that significant differences exist between type 1 and all other genetic organizations.

For 17 type strains from species (stars in **Figure 1**), which were shown to be milk-relevant in our previous study (von Neubeck et al., 2015), two strong associations were inferable: first, type strains of milk-relevant species are significantly associated to the clade consisting of the monophyletic groups *P. fluorescens*, *P. gessardii*, and *P. fragi* (Fisher's exact test, $p \sim 1.5 \times 10^{-9}$, odds-ratio ~ 39.7). In fact, only two type strains (*P. aeruginosa* DSM 50071^T and *P. protegens* CHA0^T) of species classified as milk-relevant are not members of that clade. Second, the presence of *aprX* is significantly associated to milk-relevance (Fisher's exact test, $p \sim 7.9 \times 10^{-6}$), odds-ratio ~ 25.0. Of all type strains from milk-relevant species, *Pseudomonas helleri* DSM 29165^T is the only one without the genetic information for the *aprX* locus.

Diversity of *aprX-lipA2* Genetic Compositions

The 81 type strains possessing *aprX*, as well as 86 additional strains mainly originating from raw milk samples were BLAST screened for the presence of the nine possible *aprX-lipA2* operon genes (*aprX*, *aprI*, *aprD*, *aprE*, *aprF*, *prtA*, *prtB*, *lipA1*, and *lipA2*). In total, 22 different *aprX-lipA2* genetic organizations were found among the investigated strains (**Supplementary Table S9**), including cases in which particular genes or gene cluster are detached from the operon and translocated to other positions in the genome (**Figure 2**). With the exception of *P. saponiphila* DSM 9751^T, all strains containing *aprX* are carrying the genetic information for *aprI*, *aprD*, *aprE*, and *aprF* as well. In more than 95% of cases, all five genes are lying in close proximity to each other. Therefore, these genes can be considered stable core components of the operon across species boundaries. In contrast, the remaining four genes (*prtA*, *prtB*, *lipA1*, and *lipA2*) are much more variable with respect to their presence and order in the operon and may even occur at different genomic locations or be completely absent. Mostly, strains belonging to the same species exhibit the same combination of *aprX-lipA2* genes in identical gene clusters. There are only a few cases, in which members of the species *P. lundensis*, *P. fragi*, and *Pseudomonas* sp. 12 have varying *aprX-lipA2* genetic compositions (*P. lundensis* WS 5095 and *Pseudomonas* sp. DSM 29142, *P. fragi* B25^T, *P. fragi* WS 5087, *P. fragi* WS 5094, and *P. fragi* WS 5120). **Figure 3** illustrates the four most abundant genetic organizations (type 1–4) observed in the genus, namely *aprXIDEF prtAB lipA2* (28 species), *aprXIDEF lipA2* (13 species), *aprXIDEF | prtAB* (11 species), and *aprXIDEF prtAB lipA1A2* (eight species). Together they account for more than two-thirds of all species that contain the operon or at least parts of it (**Supplementary Table S9**).

Correlation Between Peptidase Activity and *aprX-lipA2* Variants

In order to study the relationship between *aprX-lipA2* operon structure and peptidase production, the proteolytic activity of 129 *Pseudomonas* strains possessing diverse operon types was screened after 3, 4, and 7 days of growth by using an agar diffusion assay. Besides 46 type strains, the strain set contained 83 additional isolates, mainly from raw milk (**Supplementary Table S2**).

The strains split into 88 isolates exhibiting proteolysis on skim milk agar and 41 strains for which no clearing zone was observable. The activity of all proteolytic strains, except for *P. deceptionensis* DSM 26521^T, increased over time, although this rise was strongly species- and partly strain-dependent (**Figure 2**). In general, the following trends for correlating the most common operon variants (type 1–4) with proteolytic activity became apparent. As expected, all 19 tested strains missing the *aprX-lipA2* genes were non-proteolytic. In contrast, 43 isolates harboring the operon of type 1 (*aprXIDEF prtAB lipA2*) revealed the strongest proteolytic activity in our study. Mean values were significantly higher than those of strains with genetic organizations of type 2, 3, and 4 (**Figure 4**).

Moreover, for operon type 1 candidates, a distinction between members of the *P. gessardii* and *P. fluorescens* group was observed: In case of the *P. gessardii* group, 74% of strains were already highly proteolytic on day 3 and 96% showed very high proteolytic activity on day 7. In the *P. fluorescens* group, 62% of the members were moderately proteolytic on day 3 and only 53% very highly proteolytic on day 7 (**Figure 2**). Additionally, five out of six *P. lactis* and *Pseudomonas* sp. 12 strains with operon type 9, which differs from structure type 1 only by an additional *lipA1* gene in the chromosome, were strongly proteolytic on day 7. Finally, also isolates of *P. protegens*, carrying *lipA1* upstream of *prtAB* (type 12), showed high or very high proteolytic activity.

In contrast to the highly proteolytic strains, isolates with the *prtAB*-lacking operon structure *aprXIDEF lipA2* (type 2), in general, had the lowest peptidase activities in our study. However, for the 23-type 2-member two major groups could be discriminated according to their phylogenetic distance and proteolytic behavior. While all 13 isolates of *P. fragi*, *P. deceptionensis*, *P. lundensis*, and *Pseudomonas* sp. 2, belonging to the *P. fragi* subgroup, displayed no to moderate proteolytic activity, the nine tested members of *P. veronii*, *P. grimontii*, *P. marginalis*, and *P. panacis*, which are part of the *P. fluorescens* subgroup, were mostly highly proteolytic at day 7.

Remarkably, the five tested isolates with the type 3 structure (*aprXIDEF* | *prtAB*), which have the genes *prtAB* located outside of the *aprX-lipA2* gene cluster, showed very diverse proteolytic potentials on skim milk agar, ranging from no to high enzyme activity at day 7, even for three very closely related strains of the *P. fragi* subgroup (**Figure 2**). However, for members of *P. lundensis* and *P. weihenstephanensis* with operon type 8, having also the *prtAB* genes located separately plus an additional *lipA2* gene downstream of *aprF*, the majority (9 out of 13 strains) did not show any proteolytic activity.

Finally, seven strains of the *P. chlororaphis*, *P. koreensis*, and *P. mandelii* subgroups, possessing the type 4 operon (*aprXIDEF prtAB lipA1A2*) with an additional lipase gene (*lipA1*) downstream of *prtAB*, revealed also highly strain-specific, but in general reduced proteolytic activities compared to isolates missing the extra lipase gene in the operon. Interestingly, several type strains (e.g., *P. aeruginosa* DSM 50071^T, *P. fluorescens* DSM 50090^T, *P. gessardii* DSM 17152^T, *P. koreensis* DSM 16610^T, *P. mandelii* DSM 17967^T, and *P. poae* DSM 14936^T), which did not originate from milk, exhibited considerably lower proteolytic activities than corresponding milk isolates, suggesting a possible influence of environmental adaptation on the proteolytic potential of single isolates.

Quantitative Analysis of Proteolytic Activity in Milk Relevant *Pseudomonas* Species

To analyze AprX production under more realistic conditions, selected strains were cultivated in milk and peptidase activity was determined using the azocasein assay. In contrast to the screening assay on skim milk agar, this method allowed a more sensitive and specific quantification of AprX activity, since cells and supernatant were separated and thus only secreted enzymes were measured. Although bacteria were cultivated at 12°C for both assays, the azocasein assay quantified the substrate turnover of extracellular proteases after incubation of 1 h at 40°C, which corresponds more closely to the temperature optimum of AprX and the storage conditions of UHT milk products in case of long transport routes. In total, 28 strains (assigned to 18 different *Pseudomonas* species) with various operon types were selected, incubated for 4 days in UHT milk and the peptidase activity was determined after day 3 and 4.

While the proteolytic activity enhanced from day 3 to 4 between 1.7- and 4-fold for most of the strains tested, the degree of increase was strongly strain-dependent (**Figure 5**). All isolates harboring genetic organizations of type 1, 9, or 12 were strongly proteolytic and thus defined to be "high producers" (EPA > 500 Δ A/h ml), largely confirming the results based on the agar diffusion assay (**Figures 4**, **5**). Among them, *P. gessardii* WS 5049 exhibited the highest measured proteolytic activity on day 4 (2,775 $\Delta A \times h^{-1} \times ml^{-1}$).

Similarly, the eight isolates with an operon structure of type 2 (*aprXIDEF lipA2*) were classified as "low producers" in the azocasein assay (**Figure 5**). Here, all type 2 members, though belonging to phylogenetically different subgroups (*P. fragi* and *P. fluorescens*), showed very similar proteolytic behavior, which was not observed so clearly in the agar diffusion assay. Moreover, in the azocasein assay, five out of seven tested strains, which have the *prtAB* genes located outside of the operon (type 3 and 8), turned out to be "moderate producers," indicating a potential role of PrtAB on peptidase activity (**Figure 5**). These findings partly differ from the results of the agar diffusion test, where most of these strains were non-proteolytic, possibly due to other assay conditions or the lower sensitivity of this method.

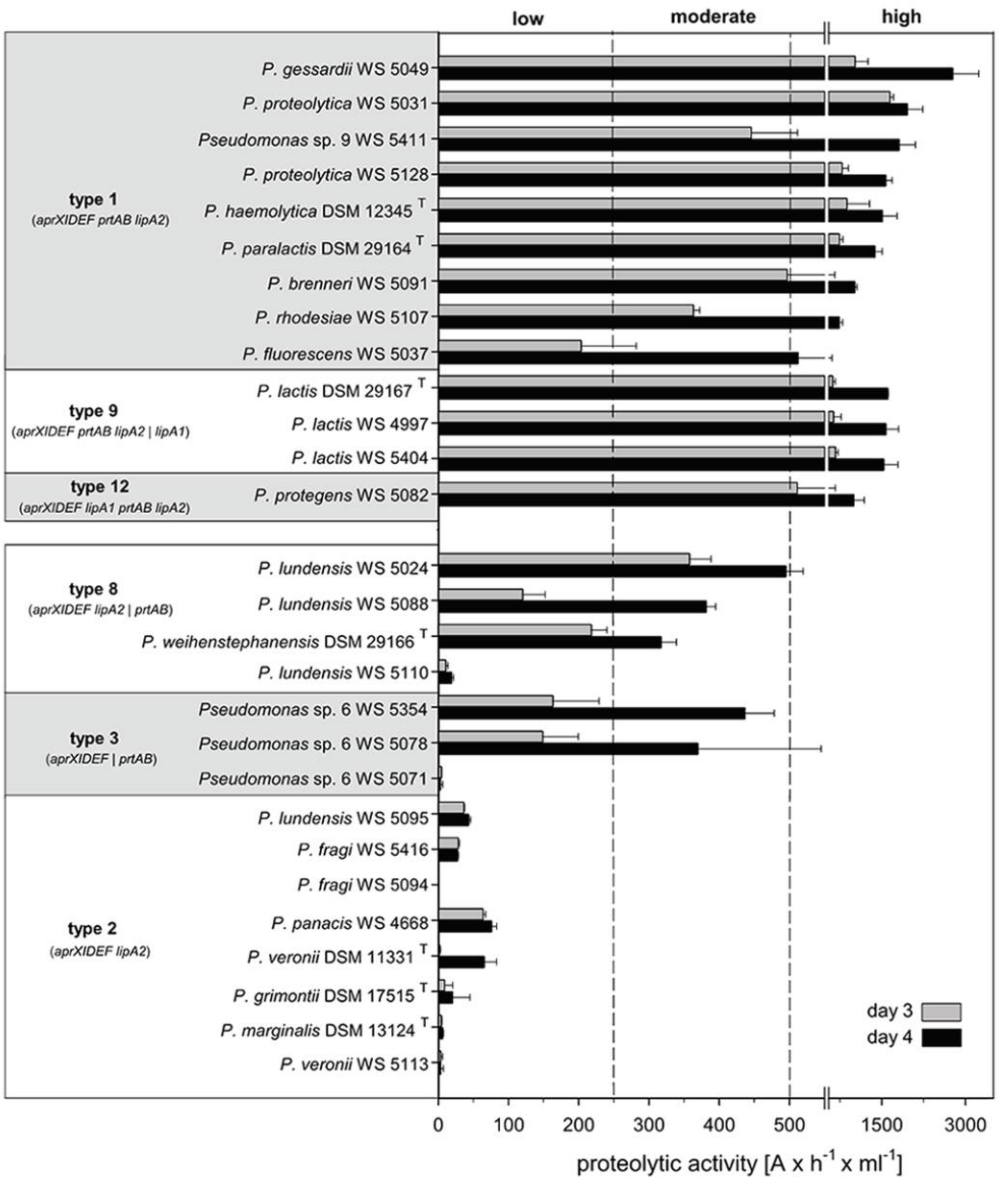

FIGURE 5 | Proteolytic activity of 28 *Pseudomonas* strains, grouped according to their *aprX-lipA2* genetic organization into highly proteolytic (type 1, 9, 12) and middle to low proteolytic isolates (type 2, 3, 8). Extracellular peptidase activity of strains, grown in UHT milk at 12°C, was determined after day 3 and 4 *via* azocasein assay. Each strain was cultivated in triplicates and EPA measurement was carried out in duplicates for each sample; means were calculated and standard deviations are shown as error bars. Dashed lines point out the classification into low, moderate and highly proteolytic isolates according to proteolytic activity values determined on day 4.

DISCUSSION

In the genus *Pseudomonas*, the expression of heat-resistant enzymes, especially the AprX peptidase, is decisive for the milk spoilage potential of strains. In this study, we assigned the presence of the *aprX*-gene to a distinct monophyletic clade comprising 88 species within the genus *Pseudomonas*. Milk relevant species were mainly allocated to the *P. gessardii*, *P. fluorescens*, and *P. fragi* subgroups. In total, 22 different *aprX-lipA2* genetic organizations were identified, substantially expanding previous work on *aprX-lipA2* operon structures in various *Pseudomonas* species. A study by Ma et al. described five different *aprX-lipA2* operon variants in six strains, which share, analogous to our findings, a set of core genes (*aprXIDEF*) and up to four additional genes (*lipA1*, *lipA2*, *prtA*, and *prtB*) (Ma et al., 2003). Our results are in good agreement, although we found only one operon type for *P. fluorescens* strains, but in total five for the whole *P. fluorescens* subgroup, while former studies revealed three different structures for the species *P. fluorescens* (Woods et al., 2001; Ma et al., 2003). These differences are likely due to difficulties in assigning *Pseudomonas* isolates to the accurate species, especially within subgroups of very closely related members.

Analysis of the proteolytic activity using agar diffusion and azocasein assay showed significant and strain-specific differences among the isolates tested, but several general correlations with the various *aprX-lipA2* genetic compositions were found. In both assays, members of the *P. gessardii* and *P. fluorescens* subgroup with a type 1 (*aprXIDEF prtAB lipA2*) or a type 9 (*aprXIDEF prtAB lipA2* | *lipA1*) operon structure reached highest proteolytic activities, while isolates containing alternative *aprX-lipA2* genetic organizations and mostly belonging to other subgroups, e.g., *P. fragi*, were much less proteolytic. This is in line with previous studies showing that proteolytic strains were frequently assigned to species of the *P. gessardii* or *P. fluorescens* subgroup and that they exhibited significantly higher enzyme activities than isolates of the *P. fragi* subgroup (Wiedmann et al., 2000; von Neubeck et al., 2015; Glück et al., 2016).

Furthermore, our study revealed that especially members of the *P. fragi* subgroup like *P. lundensis* and phylogenetically proximate species are variable in their genetic organization of the *aprX-lipA2* operon and proteolytic activity was for the most part strain-dependent. However, *via* azocasein assay, it was seen that strains of this subgroup, which possessed the *prtAB* genes apart from the *aprX-lipA2* operon (type 3, *aprXIDEF* | *prtAB* and type 8, *aprXIDEF lipA2* | *prtAB*), revealed higher proteolytic activity compared to strains lacking these genes completely (type 2, *aprXIDEF lipA2*). Therefore, and since strains with the operon structure of type 1, containing *prtAB* within the operon, were mostly highly proteolytic, a potential influence of *prtA* and *prtB* on the proteolytic activity can be suspected. The proteins PrtA (PspA) and PrtB (PspB) are described as putative autotransporters partly homologs to a serine protease found in *Serratia marcescens* (Kawai et al., 1999; Ma et al., 2003; Sun and Sun, 2015). So far, it was not shown that PrtA or PrtB themselves have peptidase activity in *Pseudomonas fluorescens*, but it was suggested that these proteins could be involved as autoaggregation factors in adhesion and are important for biofilm formation (Chabeaud et al., 2001; Woods et al., 2001). A link between the production of biofilm and extracellular enzymes has been shown before for several bacteria like *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, or *Pseudomonas* (Khajanchi et al., 2009; Teh et al., 2012; Mizan et al., 2016). However, since we measured the proteolytic activity of planktonic cells in the azocasein assay, there might be another role of PrtA and PrtB regarding AprX synthesis, independent of biofilm formation. Therefore, our further work will focus on elucidating the function of these proteins on peptidase production of *Pseudomonas* in more detail.

Besides various structures of the *aprX-lipA2* operon and a potential role of PrtAB, diverse enzyme properties of AprX itself may be another reason for variations of proteolytic activity among species assigned to different subgroups. In previous studies, a discrimination between AprX peptidases of group 1 (from *P. lundensis* and *P. weihenstephanensis* strains) and AprX of group 2 (from *P. proteolytica*, *P. lactis*, and *P. paralactis* strains) was conducted. Although sharing an amino acid sequence similarity of 68%, group 2 enzymes exhibited a 3–30-fold activity compared to peptidases of group 1 under optimal conditions for the respective enzyme (Glück et al., 2016; Volk et al., 2019). These findings support our data, revealing significant higher proteolytic activities for members of the *P. gessardii* and *P. fluorescens* subgroup (encompassing *P. proteolytica*, *P. lactis*, and *P. paralactis*) than for those of the *P. fragi* subgroup including *P. lundensis* and *P. weihenstephanensis*. Varying substrate turnover rates of AprX may also contribute to the observed higher peptidase activity from several members of the *P. fluorescens* subgroup (e.g., *P. veronii*, *P. grimontii*, *P. panacis*, and *P. marginalis*) in comparison to isolates of the *P. fragi* subgroup, though having the same operon structure of type 2. However, this effect was only observed in the agar diffusion assay, whereas in the azocasein assay both groups were not clearly distinguishable with respect to their proteolytic activity, suggesting an interplay of many factors influencing protease activity. In order to investigate further the degree of milk spoilage caused by different AprX proteins, more complex enzyme tests, using milk as substrate and detecting the release of α-amino groups for quantification of casein degradation would be necessary.

In general, inconsistent results from the two methods we used for detecting proteolytic activity occurred mainly for the non- to middle proteolytic isolates and may be due to diverse detection limits and different growth conditions, especially temperatures applied in both assays. In the literature, several environmental factors were demonstrated to affect the production or activity of AprX in *Pseudomonas* strains, including temperature (Burger et al., 2000; Nicodeme et al., 2005; Meng et al., 2018), medium composition (Martins et al., 2014), iron content (Woods et al., 2001; Maunsell et al., 2006), and calcium concentration (Ertan et al., 2015; for review see Zhang et al., 2019). Recent studies stated that temperature and pH-value have a major influence on the substrate turnover rates of AprX and showed that the optimal temperature- and pH-values vary greatly for distinct peptidases isolated from different *Pseudomonas* species, even partly independent from their phylogenetic position (Baur et al., 2015b; Glück et al., 2016; Volk et al., 2019). In addition, several works identified *aprX* as a target gene of the global GacS/GacA two-component system, a main regulator of secondary

metabolism in *Pseudomonas* (Blumer et al., 1999; Heeb et al., 2002; Hassan et al., 2010), emphasizing once more a multifactorial network controlling peptidase production.

In summary, our study demonstrates a large genetic variety of *aprX-lipA2* genetic organizations in the genus *Pseudomonas* and indicates a correlation between the proteolytic activity, the *aprX-lipA2* operon structure and the phylogenetic position of the producer. Besides such intrinsic factors, several external conditions seem to influence synthesis and activity of AprX causing strain-specific proteolytic profiles. The underlying regulatory mechanisms appear to be rather complex and need to be elucidated in future studies for a better understanding and prediction of peptidase production in *Pseudomonas*, especially for milk-relevant *Pseudomonas* species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in NCBI under PRJNA612806.

AUTHOR CONTRIBUTIONS

CM: library preparation, genome sequencing, agar diffusion assays, azocasein assays, data analysis and visualization, manuscript drafting, and writing. CH: library preparation, genome sequencing, pyhlogenomic analysis, bioinformatics analysis, statistical analysis, data analysis and visualization, manuscript drafting, and writing. MN: library preparation, genome sequencing, and data analysis. SS: data discussion, manuscript revision. MW: data discussion, manuscript revision, project planning. GL: data discussion, manuscript revision, project supervision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01190/full#supplementary-material.

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Conflict of Interest: MN is currently employed by the company Eurofins BioPharma Product Testing Munich GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary data

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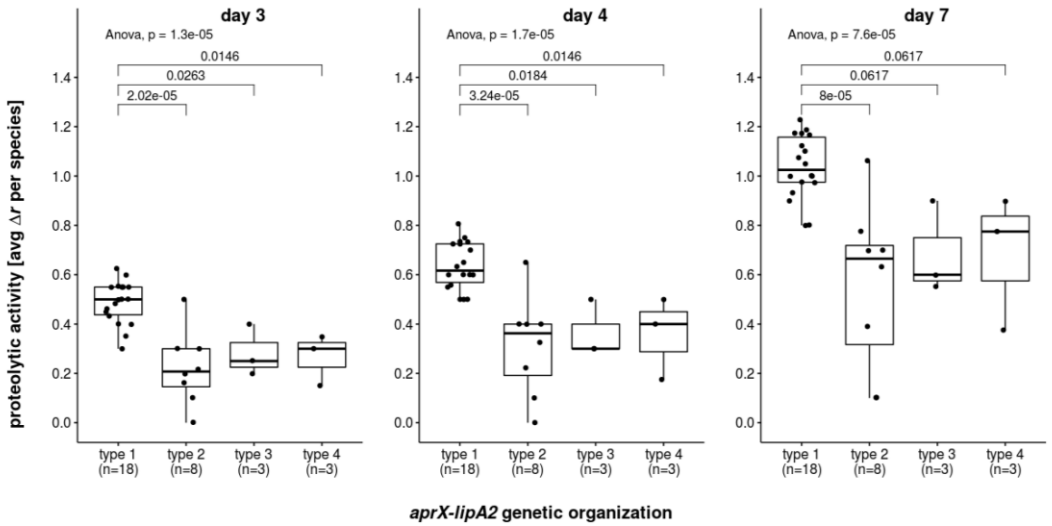
Supplementary Material

1 Supplementary Figures and Tables

1.1 Supplementary Figures



Supplementary Figure 1. Species-specific proteolytic activities at days 3, 4 and 7 plotted in dependence on the most abundant *aprX-lipA2* genetic organizations, namely *aprXIDEF prtAB lipA2* (type 1), *aprXIDEF lipA2* (type 2), *aprXIDEF* | *prtAB* (type 3) and *aprXIDEF prtAB lipA1A2* (type 4). One-way ANOVA statistical testing revealed a significant difference in mean proteolytic activity at each day. P-values resulting from post hoc tests (Tukey HSD) are shown for *aprXIDEF prtAB lipA2* (type 1) and the remaining genetic organizations.

1.2 Supplementary Tables

Supplementary Table 1. *De novo* sequenced and assembled strains

Strain	sequenced read length [bp]	high-quality read length [bp]	sequencing depth [x-fold]	N50	number contigs	assembly size	NCBI WGS ID
P. aeruginosa WS 5022	2x251 & 2x251	2x240 & 2x190	142	345,583	68	6,875,522	JAAQZH01
P. brenneri WS 5091	2x251	2x240	100	490,121	36	6,334,542	JAAQZG01
P. chengduensis DSM 26382 ^{T}	2x251	2x240	116	267,193	59	5,445,831	JAAQZF01
P. chlororaphis WS 5014	2x251	2x240	39	1,036,757	31	6,787,621	JAAQZE01
P. fluorescens WS 5037	2x251	2x150	47	392,749	48	6,247,579	JAAQZD01
P. fragi WS 5025	2x251 & 2x251	2x240 & 2x190	210	559,105	12	4,993,846	JAAQZC01
P. fragi WS 5032	2x251 & 2x251	2x190 & 2x190	76	401,936	26	4,930,841	JAAQZB01
P. fragi WS 5045	2x251 & 2x251	2x190 & 2x190	81	573,481	17	5,025,573	JAAQZA01
P. fragi WS 5065	2x251 & 2x251	2x240 & 2x190	223	585,265	18	4,907,068	JAAQYZ01
P. fragi WS 5087	2x251 & 2x251	2x190 & 2x190	76	153,202	84	5,387,582	JAAQYY01
P. fragi WS 5094	2x251 & 2x251	2x190 & 2x190	62	123,552	97	5,333,538	JAAQYX01
P. fragi WS 5102	2x251 & 2x251	2x190 & 2x190	115	428,283	38	5,496,051	JAAQYW01
P. fragi WS 5112	2x251 & 2x251	2x190 & 2x190	69	378,318	55	5,456,846	JAAQYV01
P. fragi WS 5120	2x251	2x240	107	169,642	96	5,520,514	JAAQYU01
P. fragi WS 5123	2x251	2x240	101	761,139	13	4,814,352	JAAQYT01
P. fragi WS 5124	2x251	2x240	191	559,756	18	5,127,606	JAAQYS01
P. fragi WS 5125	2x251	2x240	57	823,015	27	4,973,746	JAAQYR01
P. fragi WS 5416	2x226	2x215	53	2,817,414	12	4,997,423	JAAQYQ01
P. gessardii WS 5409	2x251	2x240	136	90,666	183	6,933,365	JAAQYP01
P. gessardii WS 5049	2x251	2x240	100	169,792	89	6,737,829	JAAQYO01
P. gessardii WS 5408	2x226	2x215	26	112,571	125	6,660,153	JAAQYN01
P. koreensis DSM 16610 ^{T}	2x251	2x240	100	689,935	27	6,086,647	JAAQYM01
P. koreensis WS 5015	2x251	2x240	42	892,370	17	6,035,452	JAAQYL01
P. lactis WS 4997	2x251	2x240	58	584,278	29	6,670,265	JAAQYK01
P. lactis WS 5000	2x226	2x215	62	892,675	26	6,536,092	JAAQYJ01
P. lactis WS 5404	2x275	2x225	80	392,731	46	6,852,845	JAAQYI01
P. lactis WS 5405	2x275	2x225	76	404,422	55	6,829,825	JAAQYH01
P. lundensis WS 5024	2x251	2x150	84	158,609	102	5,219,803	JAAQYG01
P. lundensis WS 5026	2x251	2x240	99	220,333	80	5,220,964	JAAQYF01
P. lundensis WS 5081	2x251 & 2x226	2x240 & 2x170	77	212,616	159	5,215,212	JAAQYE01
P. lundensis WS 5088	2x251	2x150	94	250,066	59	5,116,623	JAAQYD01
P. lundensis WS 5095	2x251	2x240	39	108,496	124	5,042,919	JAAQYC01
P. lundensis WS 5104	2x251	2x240	60	186,060	70	5,171,777	JAAQYB01
P. lundensis WS 5110	2x251 & 2x251	2x240 & 2x150	40	196,975	80	5,190,045	JAAQYA01
P. lundensis WS 5117	2x251	2x240	92	227,678	49	4,908,174	JAAQXZ01
P. lundensis WS 5118	2x251	2x240	123	159,291	86	5,091,333	JAAQXY01

Strain	sequenced read length [bp]	high-quality read length [bp]	sequencing depth [x-fold]	N50	number contigs	assembly size	NCBI WGS ID
P. lundensis WS 5119	2x251	2x240	132	184,907	95	5,409,286	JAAQXX01
P. lundensis WS 5121	2x251	2x240	125	325,341	58	5,007,675	JAAQXW01
P. mandelii WS 5114	2x226 & 2x251	2x170 & 2x150	68	690,265	35	6,818,060	JAAQXV01
P. nitroreducens DSM 14399 ^{T}	2x251	2x240	138	855,214	50	6,171,316	JAAQXU01
P. nitroreducens WS 5012	2x251	2x240	100	449,658	43	6,757,787	JAAQXT01
P. oryzihabitans DSM 6835 ^{T}	2x251 & 2x251	2x240 & 2x240	103	832,289	13	5,038,716	JAAQXS01
P. oryzihabitans WS 5017	2x251	2x150	60	1,068,312	19	4,995,228	JAAQXR01
P. panacis WS 4668	2x200	2x189	95	404,894	37	6,730,220	JAARME01
P. peli DSM 17833 ^{T}	2x251	2x240	100	2,500,221	15	4,511,138	JAAQXQ01
P. poae WS 5103	2x251	2x230	30	322,592	41	5,528,914	JAAQXP01
P. protegens WS 5082	2x251	2x150	46	726,802	20	6,926,774	JAARMD01
P. protegens WS 5415	2x251	2x240	19	773,105	29	6,936,464	JAAQXO01
P. proteolytica WS 5031	2x251	2x230	67	274,032	47	5,912,257	JAAQXN01
P. proteolytica WS 5060	2x251 & 2x251	2x230 & 2x190	61	119,534	126	6,489,653	JAAQXM01
P. proteolytica WS 5126	2x251	2x220	53	145,597	85	6,136,959	JAAQXL01
P. proteolytica WS 5127	2x251	2x220	37	260,396	52	5,960,850	JAAQXK01
P. proteolytica WS 5128	2x251	2x220	45	287,694	44	5,951,709	JAAQXJ01
P. proteolytica WS 5147	2x251 & 2x251	2x240 & 2x220	90	108,148	129	6,421,398	JAAQXI01
P. proteolytica WS 5148	2x251 & 2x251	2x240 & 2x220	113	145,786	113	6,550,737	JAAQXH01
P. psychrotolerans DSM 15758 ^{T}	2x251	2x240	48	324,503	40	6,012,013	JAAQXG01
P. rhodesiae WS 4669	2x200	2x189	117	219,939	65	5,983,759	JAAQXF01
P. rhodesiae WS 5107	2x251	2x150	67	224,025	58	5,961,510	JAAQXE01
Pseudomonas sp. 1 WS 5018	2x251	2x150	39	188,181	75	4,803,951	JAAQXD01
Pseudomonas sp. 2 WS 5013	2x226	2x170	78	710,455	10	4,453,840	JAAQXC01
Pseudomonas sp. 3 WS 5019	2x251	2x240	47	372,704	35	5,198,270	JAAQXB01
Pseudomonas sp. 4 WS 5011	2x251	2x240	100	216,844	88	4,604,264	JAAQXA01
Pseudomonas sp. 5 WS 5414	2x226	2x215	11	160,188	115	6,672,571	JAAQWZ01
Pseudomonas sp. 6 WS 5071	2x251 & 2x251	2x240 & 2x240	110	121,509	103	4,803,694	JAAQWY01
Pseudomonas sp. 6 WS 5078	2x251	2x150	96	264,864	41	4,738,897	JAAQWX01
Pseudomonas sp. 6 WS 5354	2x226	2x215	36	264,864	44	4,736,153	JAAQWW01
Pseudomonas sp. 7 WS 5051	2x251	2x240	66	345,925	38	5,142,615	JAAQWV01
Pseudomonas sp. 8 WS 5027	2x251 & 2x251	2x240 & 2x230	135	199,053	57	6,235,040	JAAQWU01
Pseudomonas sp. 8 WS 5086	2x251 & 2x226	2x190 & 2x170	59	198,962	61	6,148,335	JAAQWT01
Pseudomonas sp. 9 WS 5410	2x226	2x215	53	227,924	60	6,349,496	JAAQWS01
Pseudomonas sp. 9 WS 5411	2x226	2x215	28	197,246	70	5,861,914	JAARMC01
Pseudomonas sp. 9 WS 5412	2x251	2x240	41	223,471	71	6,171,677	JAARMB01
Pseudomonas sp. 9 WS 5413	2x251	2x240	50	194,584	85	6,421,091	JAAQWR01
Pseudomonas sp. 10 WS 5010	2x251	2x240	106	767,371	42	6,231,520	JAAQWQ01
Pseudomonas sp. 10 WS 5021	2x251	2x230	47	638,072	22	6,340,118	JAAQWP01
Pseudomonas sp. 10 WS 5059	2x251	2x230	32	620,406	28	6,195,332	JAAQWO01

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Strain	sequenced read length [bp]	high-quality read length [bp]	sequencing depth [x-fold]	N50	number contigs	assembly size	NCBI WGS ID
Pseudomonas sp. 10 WS 5079	2x251	2x230	37	473,683	35	6,328,935	JAAQWN01
Pseudomonas sp. 10 WS 5111	2x251 & 2x251	2x190 & 2x190	52	201,692	75	6,318,683	JAAQWM01
Pseudomonas sp. 11 WS 5146	2x251	2x220	33	541,250	37	6,482,218	JAAQWL01
Pseudomonas sp. 11 WS 5532	2x251 & 2x251	2x240 & 2x220	49	285,395	69	7,371,843	JAAQWK01
Pseudomonas sp. 12 WS 5407	2x275	2x225	129	173,495	99	6,301,008	JAAQWJ01
Pseudomonas sp. 12 WS 5503	2x226	2x215	52	480,432	38	6,706,265	JAAQWI01
Pseudomonas sp. 13 WS 5406	2x275	2x200	76	305,182	43	6,400,167	JAAQWH01
P. veronii DSM 16272	2x200	2x189	113	101,893	208	7,901,729	JAAQWG01
P. veronii WS 4670	2x200	2x189	115	200,719	111	7,064,515	JAAQWF01
P. veronii WS 4671	2x200	2x189	102	161,010	125	7,122,147	JAAQWE01
P. veronii WS 5113	2x251 & 2x251	2x240 & 2x150	42	217,144	77	6,847,663	JAAQWD01

^{T} – type strain

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Supplementary Table 2. *Pseudomonas* strains tested for proteolytic activity (agar diffusion assay)

strain	origin
P. aeruginosa DSM 50071 T	unknown
P. aeruginosa WS 5022	raw milk, Germany
P. asplenii DSM 17133 T	bird's nest fern *Asplenium nidus*, USA
P. azotoformans DSM 18862 T	paddies , Japan
P. brenneri DSM 15294 T	mineral water, France
P. brenneri WS 5091	raw milk, Germany
P. chengduensis DSM 26382 T	waste disposal site, China
P. chlororaphis WS 5014	raw milk, Germany
P. chlororaphis (ssp. *chlororaphis*) DSM 50083 T	plate contaminant, unkown
P. corrugata DSM 7228 T	*Lycopersicon esculentum*, UK
P. deceptionensis DSM 26521 T	marine sediment, Antarctica
P. endophytica B5 TT 44T	stem tissue of *Solanum tuberosum*, Spain
P. extremaustralis DSM 17835 T	water pond, Antarctica
P. fluorescens DSM 50090 T	pre-filter tanks, UK
P. fluorescens WS 5037	raw milk, Germany
P. fragi WS 5025	raw milk, Germany
P. fragi DSM 3456 T	surface water, Germnay
P. fragi WS 5032	raw milk, Germany
P. fragi WS 5045	raw milk, Germany
P. fragi WS 5065	raw milk, Germany
P. fragi WS 5087	raw milk, Germany
P. fragi WS 5094	raw milk, Germany
P. fragi WS 5102	raw milk, Germany
P. fragi WS 5112	raw milk, Germany
P. fragi WS 5120	raw milk, Germany
P. fragi WS 5123	raw milk, Germany
P. fragi WS 5124	water, Germany
P. fragi WS 5125	water, Germany
P. fragi WS 5416	raw milk, Germany
P. gessardii DSM 17152 T	mineral water, France
P. gessardii WS 5049	raw milk, Germany
P. gessardii WS 5408	raw milk, Germany
P. grimontii DSM 17515 T	mineral water, France
P. 'haemolytica' DSM 108988	raw milk, Germany
P. 'haemolytica' DSM 108987T	raw milk, Germany
P. helleri DSM 29165T	raw milk, Germany
P. helleri DSM 28141	raw milk, Germany
P. kilonensis DSM 13647 T	agricultural soil, Germany
P. koreensis DSM 16610 T	agricultural soil, Korea
P. koreensis WS 5015	raw milk, Germany
P. lactis DSM 29167 T	raw milk, Germany
P. lactis WS 4997	raw milk, Germany
P. lactis WS 5000	raw milk, Germany
P. lactis WS 5404	raw milk, Germany
P. lactis WS 5405	raw milk, Germany
P. libanensis DSM 17149 T	spring water, Lebanon
P. lini DSM 16768 T	rhizospheric soil, France
P. lundensis DSM 6252 T	prepacked beef, Sweden
P. lundensis WS 5024	raw milk, Germany
P. lundensis WS 5026	raw milk, Germany
P. lundensis WS 5081	raw milk, Germany
P. lundensis WS 5088	raw milk, Germany
P. lundensis WS 5095	raw milk, Germany

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strain	origin
P. lundensis WS 5104	raw milk, Germany
P. lundensis WS 5110	raw milk, Germany
P. lundensis WS 5117	raw milk, Germany
P. lundensis WS 5118	raw milk, Germany
P. lundensis WS 5119	raw milk, Germany
P. lundensis WS 5121	Tetra Pak, Germany
P. mandelii DSM 17967 T	mineral water, France
P. mandelii WS 5114	raw milk, Germany
P. marginalis DSM 13124 T	*Cichorium intybus,* USA
P. mosselii DSM 17497 T	medical specimen, France
P. nitroreducens DSM 14399 T	oil-brine, Japan
P. nitroreducens WS 5012	unknown, Germany
P. orientalis DSM 17489 T	spring water, Lebanon
P. oryzihabitans DSM 6835 T	rice paddy, Japan
P. oryzihabitans WS 5017	unknown, Germany
P. panacis WS 4668	raw milk, Germany
P. panacis DSM 18529 T	rusty root lesions of Korean ginseng, South Korea
P. paralactis DSM 29164 T	raw milk, Germany
P. peli DSM 17833 T	nitrifying enrichment culture, Belgium
P. poae DSM 14936 T	phyllosphere of grasses, Germany
P. poae WS 5103	raw milk, Germany
P. protegens CHA0T	soil suppressing black root rot of tobacco, Switzerland
P. protegens WS 5082	raw milk, Germany
P. proteolytica DSM 15321 T	cyanobacterial mat samples, Antarctica
P. proteolytica WS 5031	raw milk, Germany
P. proteolytica WS 5060	raw milk, Germany
P. proteolytica WS 5126	raw milk, Germany
P. proteolytica WS 5127	raw milk, Germany
P. proteolytica WS 5128	raw milk, Germany
P. proteolytica WS 5147	brown horseraddish, Germany
P. proteolytica WS 5148	water, Germany
P. psychrophila DSM 17535 T	coldroom for food storage, Japan
P. psychrotolerans DSM 15758 T	water under a dog's cage in a Medical Clinic for Small Animals, Austria
P. rhodesiae DSM 14020 T	mineral water, France
P. rhodesiae WS 4669	cow, Germany
P. rhodesiae WS 5107	raw milk, Germany
P. 'saxonica' DSM 108989 T	raw milk, Germany
P. 'saxonica' DSM 108990	raw milk, Germany
P. soli DSM 28043 T	soil, Spain
Pseudomonas sp. 1 WS 5018	raw milk, Germany
Pseudomonas sp. 2 WS 5013	semi-finished milk product, Germany
Pseudomonas sp. 3 WS 5019	water, Germany
Pseudomonas sp. 4 WS 5011	raw milk, Germany
Pseudomonas sp. 5 WS 5414	raw milk, Germany
Pseudomonas sp. 6 WS 5071	raw milk, Germany
Pseudomonas sp. 6 WS 5078	raw milk, Germany
Pseudomonas sp. 6 WS 5354	raw milk, Germany
Pseudomonas sp. 7 WS 5051	raw milk, Germany
Pseudomonas sp. 8 WS 5027	raw milk, Germany
Pseudomonas sp. 8 WS 5086	raw milk, Germany
Pseudomonas sp. 9 WS 5410	raw milk, Germany
Pseudomonas sp. 9 WS 5411	raw milk, Germany
Pseudomonas sp. 9 WS 5412	raw milk, Germany
Pseudomonas sp. 10 WS 5010	raw milk, Germany

strain	origin
Pseudomonas sp. 10 WS 5021	raw milk, Germany
Pseudomonas sp. 10 WS 5059	raw milk, Germany
Pseudomonas sp. 10 WS 5079	raw milk, Germany
Pseudomonas sp. 10 WS 5111	raw milk, Germany
Pseudomonas sp. 11 WS 5532	unknown
Pseudomonas sp. 11 WS 5146	milk product, Germany
Pseudomonas sp. 12 DSM 29142	raw milk, Germany
Pseudomonas sp. 12 WS 5407	raw milk, Germany
Pseudomonas sp. 12 WS 5503	raw milk, Germany
Pseudomonas sp. 13 WS 5406	raw milk, Germany
P. synxantha DSM 18928 ^{T}	cream, USA
P. syringae subsp. syringae DSM 10604 ^{T}	*Syringa vulgaris*, UK
P. taetrolens DSM 21104 ^{T}	musty egg, unknown
P. trivialis DSM 14937 ^{T}	phyllosphere of grasses, Germnay
P. veronii DSM 11331 ^{T}	mineral water, France
P. veronii DSM 16272	sediment, Netherlands
P. veronii WS 4670 ^{T}	semi-finished milk product, Germany
P. veronii WS 4671	semi-finished milk product, Germany
P. veronii WS 5113	raw milk, Germany
P. viridiflava DSM 11124 ^{T}	dwarf or runner bean, Switzerland
P. weihenstephanensis DSM 29140	raw milk, Germany
P. weihenstephanensis DSM 29166 ^{T}	raw milk, Germany

^{T} – type strain

Supplementary Table 3. Genome data of *Pseudomonas* type strains downloaded from NCBI

strain	NCBI WGS ID	RefSeq/GenBank IDs
P. abietaniphila ATCC 700689^T	FNCO01	
P. abyssi MT 5 ^{T}	NTMR01	
P. aeruginosa DSM 50071 ^{T}	JYLC01	
P. aestusnigri CECT 8317 ^{T}	FNVE01	
P. alcaligenes NBRC 14159 ^{T}	BATI01	
P. alcaliphila JCM 10630 ^{T}	FNAE01	
P. alkylphenolica KL28 ^{T}		NZ_CP009048.1
P. amygdali ICMP 3918 ^{T}	LJPQ01	
P. anguilliseptica DSM 12111 ^{T}	FNSC01	
P. antarctica DSM 15318 ^{T}	UYXQ01	
P. argentinensis LMG 22563 ^{T}	FORC01	
P. arsenicoxydans CECT 7543 ^{T}		LT629705.1
P. 'asiatica' RYU5 ^{T}		DRR138841 (SRA)
P. asplenii ATCC 23835 ^{T}		LT629777.1
P. asturiensis LMG 26898 ^{T}	FRDA01	
P. avellanae BPIC 631 ^{T}	AKBS01	
P. azotifigens DSM 17556 ^{T}	AUDU01	
P. azotoformans LMG 21611 ^{T}		NZ_LT629702.1
P. baetica LMG 25716 ^{T}	PHHE01	
P. balearica DSM 6083 ^{T}		CP007511.1
P. bauzanensis DSM 22558 ^{T}	FOGN01	
P. benzenivorans DSM 8628 ^{T}	FNCT01	
P. 'bohemica' IA19 ^{T}	NKHL01	
P. borbori DSM 17834 ^{T}	FOWX01	
P. brenneri DSM 15294 ^{T}	VFIL01	
P. caeni DSM 24390 ^{T}	ATXQ01	
P. canadensis 2-92 ^{T}	AYTD01	
P. cannabina ICMP 2823 ^{T}	FNKU01	
P. caricapapayae ICMP 2855 ^{T}	LJPW01	
P. caspiana FBF102 ^{T}	LOHF01	
P. cedrina ssp. *cedrina* DSM 17516 ^{T}	MNPW01	
P. cerasi 58 ^{T}		NZ_LT222313.1 - NZ_LT222319.1
P. chlororaphis ssp. *aurantiaca* LMG 21630 ^{T}		NZ_LT629747.1
P. chlororaphis ssp. *aureofaciens* NBRC 3521 ^{T}	BBQB01	
P. chlororaphis ssp. *chlororaphis* DSM 50083 ^{T}	VFIN01	
P. chlororaphis ssp. *piscium* DSM 21509 ^{T}	LHUZ01	
P. cichorii ATCC 10857 ^{T}	FNIK01	
P. citronellolis LMG 18378 ^{T}	FOLS01	
P. coleopterorum LMG 28558 ^{T}	FNTZ01	
P. composti CCUG 59231 ^{T}	FOWP01	
P. congelans DSM 14939 ^{T}	FNJH01	
P. corrugata DSM 7228 ^{T}	LHVK01	
P. costantinii LMG 22119 ^{T}	MDDR01	
P. cremoricolorata DSM 17059 ^{T}	AUEA01	
P. cuatrocienegasensis CIP 109853 ^{T}	FOFP01	
P. deceptionensis DSM 26521 ^{T}	JYKX01	
P. delhiensis CCM 7361 ^{T}	FNEC01	
P. donghuensis HYS ^{T}	AJJP01	
P. endophytica BSTT44 ^{T}	LLWH01	
P. entomophila L48 ^{T}		NC_008027.1
P. extremaustralis DSM 17835 ^{T}	VFET01	

strain	NCBI WGS ID	RefSeq/GenBank IDs
P. extremorientalis LMG 19695 T	MDGK01	
P. ficuserectae ICMP 7848 T	LJQJ01	
P. flavescens NBRC 103044 T	BCZZ01	
P. flexibilis CGMCC 1.1365 T	FMUP01	
P. floridensis GEV388 T	MUIO01	
P. fluorescens DSM 50090 T	VFEP01	
P. fluvialis ASS-1 T	NMQV01	
P. formosensis JCM 18415 T	FOYD01	
P. fragi B25 T	AHZX01	
P. fulva NBRC 16637 T	JHYU01	
P. furukawaii KF707 T	AJMR01	
P. fuscovaginae LMG 2158 T		NZ_LT629972.1
P. 'gallaeciensis' V113 T	LMAZ01	
P. gessardii DSM 17152 T	VFEW01	
P. 'qingdaonensis' JJ3 T	PHTD01	
P. graminis DSM 11363 T	FOHW01	
P. granadensis LMG 27940 T		LT629778.1
P. grimontii DSM 17515 T	VFES01	
P. guangdongensis CCTCC 2012022 T		LT629780.1
P. guariconensis LMG 27394 T	FMYX01	
P. guguanensis JCM 18416 T	FNJJ01	
P. guineae LMG 24016 T	FOQL01	
P. 'haemolytica' DSM 108987 T	VOIW01	
P. 'haemolytica' DSM 108988	VOIX01	
P. helleri DSM 29165 T	JYLD01	
P. helleri DSM 28141	JYLE01	
P. hussainii JCM 19513 T	FOAS01	
P. indica NBRC 103045 T	BDAC01	
P. indoloxydans JCM 14246 T	QASO01	
P. japonica NBRC 103040 T	BBIR01	
P. jessenii DSM 17150 T	NIWT01	
P. jinjuensis NBRC 103047 T	BDAD01	
P. kilonensis DSM 13647 T	LHVH01	
P. knackmussii B13 T		HG322950.1
P. 'kribbensis' 46-2 T		NZ_CP029608.1
P. kunmingensis DSM 25974 T	FORS01	
P. kuykendallii NRRL B-59562 T	FNNU01	
P. lactis DSM 29167 T	JYLO01	
P. 'laurylsulfatovorans' AP3_22 T	MUJK01	
P. libanensis DSM 17149 T	JYLH01	
P. lini DSM 16768 T	JYLB01	
P. linyingensis LMG 25967 T	FNZE01	
P. litoralis 2SM5 T	LT629748.1	
P. lundensis DSM 6252 T	JYKY01	
P. lurida LMG 21995 T	PDJB01	
P. lutea DSM 17257 T	JRMB01	
P. luteola NBRC 103146 T	BDAE01	
P. mandelii DSM 17967 T	VFIM01	
P. 'mangrovi' TC11 T	QASN01	
P. marginalis DSM 13124 T	VFEQ01	
P. marincola JCM 14761 T	FPBC01	
P. 'massiliensis' CB1 T	CCYK01	
P. mediterranea CFBP 5447 T	AUPB01	
P. meliae CFBP 3225 T	JYHE01	
P. mendocina NBRC 14162 T	BBQC01	

Supplementary Material

strain	NCBI WGS ID	RefSeq/GenBank IDs
P. migulae NBRC 103157 T	BDAG01	
P. mohnii DSM 18327 T	FNRV01	
P. monteilii NBRC 103158 T	JHYV01	
P. mosselii DSM 17497 T	JHYW01	
P. mucidolens NBRC 103159 T	BDAH01	
P. oceani DSM 100277 T	PPSK01	
P. oleovorans ssp. *lubricantis* RS1 T	FNZC01	
P. oleovorans ssp. *oleovorans* NBRC 13583 T	BDAL01	
P. orientalis DSM 17489 T	JYLM01	
P. 'oryzae' KCTC 32247 T		LT629751.1
P. otitidis DSM 17224 T	FOJP01	
P. pachastrellae CCUG 46540 T	MUBC01	
P. palleroniana LMG 23076 T	PYWX01	
P. panacis DSM 18529 T	VFER01	
P. panipatensis CCM 7469 T	FNDS01	
P. parafulva DSM 17004 T	AUEB01	
P. paralactis DSM 29164 T	JYLN01	
P. pelagia CL-AP6 T	AROI01	
P. 'pharmafabricae' ZYSR67-Z T	PIYS01	
P. plecoglossicida NBRC 103162 T	JHYX01	
P. poae DSM 14936 T	JYLI01	
P. pohangensis DSM 17875 T		LT629785.1
P. prosekii LMG 26867 T		LT629762.1
P. protegens CHA0 T		NC_021237.1
P. proteolytica DSM 15321 T	VFEV01	
P. psychrophila DSM 17535 T	JYKZ01	
P. punonensis CECT 8089 T	FRBQ01	
P. putida NBRC 14164 T		NC_021505.1
P. reidholzensis CCOS 865 T	UNOZ01	
P. reinekei MT1 T	MSTQ01	
P. resinovorans DSM 21078 T	AUIE01	
P. rhizosphaerae DSM 16299 T		CP009533.1
P. rhodesiae DSM 14020 T	VFEU01	
P. sabulinigri JCM 14963 T		LT629763.1
P. sagittaria JCM 18195 T	FOXM01	
P. 'saxonica' DSM 108989 T	VFIO01	
P. 'saxonica' DSM 108990	VFIP01	
P. salegens CECT 8338 T		LT629787.1
P. salomonii ICMP 14252 T	FNOX01	
P. saponiphila DSM 9751 T	FNTJ01	
P. savastanoi ICMP 4352 T	LJRJ01	
P. 'sediminis' PI11 T	NIQU01	
P. segetis CIP 108523 T	FZOG01	
P. seleniipraecipitans LMG 25475 T	FNBM01	
P. 'sichuanensis' WCHPs060039 T	QKVM01	
P. 'sihuiensis' KCTC 32246 T		LT629797.1
P. silesiensis A3 T		NZ_CP014870.1
P. simiae CCUG 50988 T	FOKB01	
P. soli LMG 27941 T	FOEQ01	
P. 'songnenensis' NEAU-ST5-5 T	RFFN01	
Pseudomonas sp. DSM 29142	JYLP01	
P. straminea JCM 2783 T	FOMO01	
P. stutzeri ATCC 17588 T		NC_015740.1
P. synxantha DSM 18928 T	JYLJ01	

strain	NCBI WGS ID	RefSeq/GenBank IDs
P. syringae ssp. *syringae* DSM 10604 ^{T}	JALK01	
P. taeanensis MS-3 ^{T}	AWSQ01	
P. taetrolens DSM 21104 ^{T}	JYLA01	
P. taiwanensis DSM 21245 ^{T}	AUEC01	
P. thermotolerans DSM 14292 ^{T}	AQPA01	
P. thivervalensis DSM 13194 ^{T}	LHVE01	
P. tolaasii NCPPB 2192 ^{T}		NZ_CP020369.1
P. toyotomiensis JCM 15604 ^{T}	FOXK01	
P. tremae ICMP 9151 ^{T}	LJRO01	
P. trivialis DSM 14937 ^{T}	JYLK01	
P. tuomuerensis JCM 14085 ^{T}	JTAK01	
P. umsongensis DSM 16611 ^{T}	NIWU01	
P. veronii DSM 11331 ^{T}	JYLL01	
P. versuta L10.10 ^{T}		NZ_CP012676.1
P. viridiflava DSM 6694 ^{T}	JRXH01	
P. vranovensis DSM 16006 ^{T}	AUED01	
P. wadenswilerensis CCOS 864 ^{T}	UIDD01	
P. weihenstephanensis DSM 29166 ^{T}	JYLF01	
P. weihenstephanensis DSM 29140	JYLG01	
P. xanthomarina DSM 18231 ^{T}	FQXA01	
P. xinjiangensis NRRL B-51270 ^{T}		NZ_LT629736.1
P. yamanorum LMG 27247 ^{T}		LT629793.1
P. zeshuii KACC 15471 ^{T}	FQYS01	
P. zhaodongensis NEAU-ST5-21 ^{T}	RFFM01	

^{T} – **type strain**

Supplementary Table 4. Shapiro-Wilk and Levene tests on agar diffusion assay data

statistical test	p-values		
	day 3	day 4	day 7
Levene's test (homogeneity of variance)	0.5989	0.3050	0.1456
Shapiro-Wilk test (normality of data)	0.7732	0.6371	0.2840

Supplementary Table 5. Validly described *Pseudomonas* species/subspecies (LPSN as of March 2019)

species number	species/subspecies (reference[LPSN])	species number	species/subspecies (reference[LPSN])
1	*P. abietaniphila* (Mohn *et al.*1999)	93	*P. litoralis* (Pascual *et al.* 2012)
2	*P. abyssi* (Wei *et al.* 2018)	94	*P. lundensis* (Molin *et al.* 1986)
3	*P. aeruginosa* (Schroeter 1872)	95	*P. lurida* (Behrendt *et al.* 2007)
4	*P. aestusnigri* (Sanchez *et al.* 2014)	96	*P. lutea* (Peix *et al.* 2004)
5	*P. agarici* (Young 1970)	97	*P. luteola* (Kodama *et al.* 1985)
6	*P. alcaligenes* (Monias 1928)	98	*P. mandelii* (Verhille *et al.* 1999)
7	*P. alcaliphila* (Yumoto *et al.* 2001)	99	*P. marginalis* (Brown 1918)
8	*P. alkylphenolica* (Mulet *et al.* 2015)	100	*P. marincola* (Romanenko *et al.* 2008)
9	*P. amygdali* (Psallidas & Panagopoulos 1975)	101	*P. matsuisoli* (Lin *et al.* 2015)
10	*P. anguilliseptica* (Wakabayashi & Egusa 1972)	102	*P. mediterranea* (Catara *et al.* 2002)
11	*P. antarctica* (Reddy *et al.* 2004)	103	*P. meliae* (Ogimi 1981)
12	*P. argentinensis* (Peix *et al.* 2005)	104	*P. mendocina* (Palleroni 1970)
13	*P. arsenicoxydans* (Campos *et al.* 2011)	105	*P. meridian* (Reddy *et al.* 2004)
14	*P. asplenii* (Ark & Tompkins 1946)	106	*P. migulae* (Verhille *et al.* 1999)
15	*P. asturiensis* (González *et al.* 2013)	107	*P. mohnii* (Cámara *et al.* 2007)
16	*P. asuensis* (Reddy & Garcia-Pichel 2015)	108	*P. monteilii* (Elomari *et al.* 1997)
17	*P. avellanae* (Janse *et al.* 1997)	109	*P. moorei* (Cámara *et al.* 2007)
18	*P. azotifigens* (Hatayama *et al.* 2005)	110	*P. moraviensis* (Tvrzová *et al.* 2006)
19	*P. azotoformans* (Iizuka & Komagata 1963)	111	*P. mosselii* (Dabboussi *et al.* 2002)
20	*P. baetica* (López *et al.* 2012)	112	*P. mucidolens* (Levine & Anderson 1932)
21	*P. balearica* (Bennasar *et al.* 1996)	113	*P. nitritireducens* (Wang *et al.* 2015)
22	*P. bauzanensis* (Zhang *et al.* 2011)	114	*P. nitroreducens* (Iizuka & Komagata 1964)
23	*P. benzenivorans* (Lang *et al.* 2012)	115	*P. oceani* (Wang & Sun 2016)
24	*P. borbori* (Vanparys *et al.* 2006)	116	*P. oleovorans* ssp. *lubricantis* (Saha *et al.* 2010)
25	*P. brassicacearum ssp. brassicacearum* (Achouak *et al.* 2000)	116	*P. oleovorans* ssp. *oleovorans* (Lee & Chandler 1941)
25	*P. brassicacearum ssp. neoaurantiaca* (Ivanova *et al.* 2009)	117	*P. orientalis* (Dabboussi *et al.* 2002)
26	*P. brenneri* (Baïda *et al.* 2002)	118	*P. oryzihabitans* (Kodama *et al.* 1985)
27	*P. caeni* (Xiao *et al.* 2009)	119	*P. otitidis* (Clark *et al.* 2006)
28	*P. canadensis* (Tambong *et al.* 2017)	120	*P. pachastrellae* (Romanenko *et al.* 2005)
29	*P. cannabina* (ex Šutič & Dowson 1959)	121	*P. palleroniana* (Gardan *et al.* 2002)
30	*P. caricapapayae* (Robbs 1956)	122	*P. panacis* (Park *et al.* 2005)
31	*P. caspiana* (Busquets *et al.* 2017)	123	*P. panipatensis* (Gupta *et al.* 2008)
32	*P. cedrina* ssp. *cedrina* (Dabboussi *et al.* 2002)	124	*P. parafulva* (Uchino *et al.* 2002)
32	*P. cedrina* ssp. *fulgida* (Behrendt *et al.* 2009)	125	*P. paralactis* (von Neubeck *et al.* 2017)
33	*P. cerasi* (Kaluzna *et al.* 2017)	126	*P. pelagia* (Hwang *et al.* 2009)
34	*P. chengduensis* (Tao *et al.* 2014)	127	*P. peli* (Vanparys *et al.* 2006)
35	*P. chlororaphis* ssp. *aurantiaca* (Nakhimovskaya 1948)	128	*P. pertucinogena* (Kawai & Yabuuchi 1975)
35	*P. chlororaphis* ssp. *aureofaciens* (Kluyver 1956)	129	*P. plecoglossicida* (Nishimori *et al.* 2000)
35	*P. chlororaphis* ssp. *chlororaphis* (Guignard & Sauvageau 1894)	130	*P. poae* (Behrendt *et al.* 2003)
35	*P. chlororaphis* ssp. *piscium* (Burr *et al.* 2010)	131	*P. pohangensis* (Weon *et al.* 2006)
36	*P. cichorii* (Swingle 1925)	132	*P. populi* (Anwar *et al.* 2016)
37	*P. cissicola* (Takimoto 1939)	133	*P. profundi* (Sun *et al.* 2018)
38	*P. citronellolis* (Seubert 1960)	134	*P. prosekii* (Kosina *et al.* 2014)
39	*P. coleopterorum* (Menéndez *et al.* 2015)	135	*P. protegens* (Ramette *et al.* 2012)
40	*P. composti* (Gibello *et al.* 2011)	136	*P. proteolytica* (Reddy *et al.* 2004)
41	*P. congelans* (Behrendt *et al.* 2003)	137	*P. psychrophila* (Yumoto *et al.* 2002)
42	*P. corrugata* (Roberts & Scarlett 1981)	138	*P. psychrotolerans* (Hauser *et al.* 2004)
43	*P. costantinii* (Munsch *et al.* 2002)	139	*P. gunonensis* (Ramos *et al.* 2013)
44	*P. cremoricolorata* (Uchino *et al.* 2002)	140	*P. putida* (Trevisan 1889)
45	*P. cuatrocienegasensis* (Escalante *et al.* 2009)	141	*P. reidholzensis* (Frasson *et al.* 2017)
46	*P. deceptionensis* (Carrión *et al.* 2011)	142	*P. reinekei* (Cámara *et al.* 2007)

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47	*P. delhiensis* (Prakash *et al.* 2007)	143	*P. resinovorans* (Delaporte *et al.* 1961)
48	*P. donghuensis* (Gao *et al.* 2015)	144	*P. rhizosphaerae* (Peix *et al.* 2003)
49	*P. duriflava* (Liu *et al.* 2008)	145	*P. rhodesiae* (Coroler *et al.* 1997)
50	*P. endophytica* (Ramírez-Bahena *et al.* 2015)	146	*P. sabulinigri* (Kim *et al.* 2009)
51	*P. entomophila* (Mulet *et al.* 2012)	147	*P. sagittaria* (Lin *et al.* 2013)
52	*P. extremaustralis* (López *et al.* 2010)	148	*P. salegens* (Amoozegar *et al.* 2014)
53	*P. extremorientalis* (Ivanova *et al.* 2002)	149	*P. salina* (Zhong *et al.* 2015)
54	*P. ficuserectae* (Goto 1983)	150	*P. salomonii* (Gardan *et al.* 2002)
55	*P. flavescens* (Hildebrand *et al.* 1994)	151	*P. saponiphila* (Lang *et al.* 2012)
56	*P. flexibilis* (Shin *et al.* 2016)	152	*P. savastanoi* (Janse 1982)
57	*P. floridensis* (Timilsina *et al.* 2018)	153	*P. segetis* (Park *et al.* 2006)
58	*P. fluorescens* (Migula 1895)	154	*P. seleniipraecipitans* (Hunter & Manter 2011)
59	*P. fluvialis* (Sudan *et al.* 2018)	155	*P. sesami* (Madhaiyan *et al.* 2017)
60	*P. formosensis* (Lin *et al.* 2013)	156	*P. silesiensis* (Kaminski *et al.* 2018)
61	*P. fragi* (Gruber 1905)	157	*P. simiae* (Vela *et al.* 2006)
62	*P. frederiksbergensis* (Andersen *et al.* 2000)	158	*P. soli* (Pascual *et al.* 2015)
63	*P. fulva* (Iizuka & Komagata 1963)	159	*P. straminea* (Iizuka & Komagata 1963)
64	*P. furukawaii* (Kimura *et al.* 2018)	160	*P. stutzeri* (Lehmann & Neumann 1896
65	*P. fuscovaginae (ex* Tanii *et al.* 1976)	161	*P. synxantha* (Ehrenberg 1840)
66	*P. gelidicola* (Kadota 1951)	162	*P. syringae* ssp. *syringae* (van Hall 1902)
67	*P. gessardii* (Verhille *et al.* 1999)	163	*P. taeanensis* (Lee *et al.* 2010)
68	*P. glareae* (Romanenko *et al.* 2015)	164	*P. taetrolens* (Haynes 1957)
69	*P. graminis* (Behrendt *et al.* 1999)	165	*P. taiwanensis* (Wang *et al.* 2010)
70	*P. granadensis* (Pascual *et al.* 2015)	166	*P. tarimensis* (Anwar *et al.* 2017)
71	*P. grimontii* (Baïda *et al.* 2002)	167	*P. thermotolerans* (Manaia & Moore 2002)
72	*P. guangdongensis* (Yang *et al.* 2013)	168	*P. thivervalensis* (Achouak *et al.* 2000)
73	*P. guariconensis* (Toro *et al.* 2013)	169	*P. tolaasii* (Paine 1919)
74	*P. guguanensis* (Liu *et al.* 2013)	170	*P. toyotomiensis* (Hirota *et al.* 2011)
75	*P. guineae* (Bozal *et al.* 2007)	171	*P. tremae* (Gardan *et al.* 1999)
76	*P. helleri* (von Neubeck *et al.* 2016)	172	*P. trivialis* (Behrendt *et al.* 2003)
77	*P. helmanticensis* (Ramírez *et al.* 2014)	173	*P. tuomuerensis* (Xin *et al.* 2009)
78	*P. hussainii* (Hameed *et al.* 2014)	174	*P. turukhanskensis* (Korshunova *et al.* 2016)
79	*P. indica* (Pandey *et al.* 2002)	175	*P. umsongensis* (Kwon *et al.* 2003)
80	*P. indoloxydans* (Gray 1928)	176	*P. vancouverensis* (Mohn *et al.* 1999)
81	*P. japonica* (Pungrasmi *et al.* 2008)	177	*P. veronii* (Elomari *et al.* 1996)
82	*P. jessenii* (Verhille *et al.* 1999)	178	*P. versuta* (See *et al.* 2017)
83	*P. jinjuensis* (Kwon *et al.* 2003)	179	*P. viridiflava* (Burkholder 1930)
84	*P. kilonensis* (Sikorski *et al.* 2001)	180	*P. vranovensis* (Tvrzová *et al.* 2006)
85	*P. knackmussii* (Stolz *et al.* 2007)	181	*P. wadenswilerensis* (Frasson *et al.* 2017)
86	*P. koreensis* (Kwon *et al.* 2003)	182	*P. weihenstephanensis* (von Neubeck *et al.* 2016)
87	*P. kunmingensis* (Xie *et al.* 2014)	183	*P. xanthomarina* (Romanenko *et al.* 2005)
88	*P. kuykendallii* (Hunter & Manter 2012)	184	*P. xiamenensis* (Lai & Shao 2008)
89	*P. lactis* (von Neubeck *et al.* 2017)	185	*P. xinjiangensis* (Liu *et al.* 2009)
90	*P. libanensis* (Dabboussi *et al.* 1999)	186	*P. yamanorum* (Arnau *et al.* 2015)
91	*P. lini* (Delorme *et al.* 2002)	187	*P. zeshuii* (Feng *et al.* 2012)
92	*P. linyingensis* (He *et al.* 2015)	188	*P. zhaodongensis* (Zhang *et al.* 2015)

reference^{LPSN} – reference according to LSPN (http://www.bacterio.net)

Supplementary Table 6. Phylogenomic location of type strains not considered in previous MLSA analyses

monophyletic group	newly added type strain
P. pertucinogena	*P. abyssi* MT 5, *P. 'gallaeciensis'* V113, *P. oceani* DSM 100277
P. alcaligenes (new group)	*P. fluvialis* ASS-1, *P. 'pharmafabricae'* ZYSR67-Z
P. oleovorans	*P. oleovorans* ssp. *lubricantis* RS1, *P. 'sediminis'* PI11
P. stutzeri	*P. 'songnenensis'* NEAU-ST5-5, *P. zhaodongensis* NEAU-ST5-21
P. aeruginosa	*P. furukawaii* KF707
P. putida	*P. 'asiatica'* RYU5, *P. 'sichuanensis'* WCHPs060039, *P. 'qingdaonensis'* JJ3
P. lutea	*P. 'bohemica'* IA19
P. syringae	*P. floridensis* GEV388
P. chlororaphis	*P. chlororaphis* ssp. *piscium* DSM 21509
P. jessenii	*P. 'laurylsulfatovorans'* AP3_22
P. mandelii	*P. silesiensis* A3
P. fragi	*P. 'saxonica'* DSM 108989
P. fluorescens	*P. 'haemolytica'* DSM 108987
no affiliation	*P. hussainii* JCM 19513, *P. 'mangrovi'* TC11, *P. pohangensis* DSM 17875

Supplementary Table 7. *Pseudomonas* type strains carrying mutations in the *aprX* gene

type strain	mutation/abnormality
P. lundensis DSM 6252	only partially present due to end of contig
P. savastanoi ICMP 4352	frame-shift mutation due to 4-bp deletion
P. meliae CFBP 3225	frame-shift mutation due to 16-bp deletion
P. ficuserectae ICMP 7848	nonsense mutation (premature stop codon)
P. cannabina ICMP 2823	coding sequence splitted into 2 parts (5' and 3') due to >600-bp insertion

Supplementary Table 8. Frequency of occurrence of the *aprX* locus on the level of monophyletic groups

monophyletic group	number of type strains	frequency of type strains containing *aprX*
P. fluorescens	26	96.2 %
P. gessardii	4	100 %
P. fragi	10	50 %
P. mandelii	6	100 %
P. jessenii	5	20 %
P. koreensis	4	100 %
P. corrugata	4	100 %
P. chlororaphis	6	100 %
P. asplenii	2	100 %
P. syringae	16	87.5 %
P. lutea	4	20 %
P. rhizosphaerae	2	0 %
P. putida	20	30 %
P. anguilliseptica	9	0 %
P. straminae	5	0 %
P. aeruginosa	17	5.9 %
P. stutzeri	7	0 %
P. oleovorans	11	0 %
P. alcaligenes	3	0 %
P. luteola	2	0 %
P. oryzihabitans	2	0 %
P. pertucinogena	12	0 %

3 Chapter 2: "Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR"

Summary

Psychrotolerant *Pseudomonas* species can dominate the microbiota of refrigerated raw milk. Although these pseudomonads secrete only the same caseinolytic peptidase, the metallopeptidase AprX, different species vary considerably in their proteolytic potential.

In this study, two triplex qPCR assays were established to detect proteolytic and frequent *Pseudomonas* species in raw milk. Therefore, five species-specific hydrolysis probes, binding the *aprX* gene, were designed to quantify highly proteolytic (*P. proteolytica*, *P. gessardii*, *P. lactis*, *P. fluorescens*, *P. protegens*) and moderate to low caseinolytic species (*P. lundensis* and *P. fragi*). Moreover, an additional universal *Pseudomonas* probe, binding *rpoB*, was created to enumerate the total *Pseudomonas* counts. Quantitative PCR, in singleplex and multiplex approach, resulted in regression lines between DNA concentrations and C_q values with R^2-values >0.975, and amplification efficiencies between 85–97% for all six hydrolysis probes and primer pairs. Besides, high specificity was determined by applying the DNA of 75 target and non-target *Pseudomonas* spp., allocated to 57 distinct species, and of strains from 40 other bacteria. In addition, a broad detection range between 10^3–10^7 cfu/ml and high sensitivity of at least 2×10^2–2×10^3 cfu/ml, depending on the respective probe, were received for the qPCR assay by applying DNA, which was extracted from artificially inoculated raw milk, as a template. Moreover, total *Pseudomonas* counts determined for 60 raw milk samples were mainly consistent when comparing classical cultivation on selective agar with the newly developed qPCR method using the universal *Pseudomonas* probe. Interestingly, each of the 60 raw milk samples revealed a unique composition of the *Pseudomonas* population, and *P. lundensis*, *P. proteolytica,* and *P. gessardii* were identified as the most frequently occurring species.

Thus, the two triplex qPCR assays present not only a solid alternative to selective cultivation for the determination of *Pseudomonas* counts as it is less time-consuming and exhibits a higher specificity. Furthermore, it allows the quantification of distinct *Pseudomonas* species according to their proteolytic potential and could present a valuable tool to assess the quality of raw milk before further processing.

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METHODS AND PROTOCOLS

Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR

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Abstract
The heat-stable peptidase AprX, secreted by psychrotolerant *Pseudomonas* species in raw milk, is a major cause of destabilization and premature spoilage of ultra-high temperature (UHT) milk and milk products. To enable rapid detection and quantification of seven frequent and proteolytic *Pseudomonas* species (*P. proteolytica*, *P. gessardii*, *P. lactis*, *P. fluorescens*, *P. protegens*, *P. lundensis*, and *P. fragi*) in raw milk, we developed two triplex qPCR assays taking into account species-dependent differences in AprX activity. Besides five species-specific hydrolysis probes, targeting the *aprX* gene, a universal *rpoB* probe was included in the assay to determine the total *Pseudomonas* counts. For all six probes, linear regression lines between C_q value and target DNA concentration were obtained in singleplex as well as in multiplex approaches, yielding R^2 values of > 0.975 and amplification efficiencies of 85–97%. Moreover, high specificity was determined using genomic DNA of 75 *Pseudomonas* strains, assigned to 57 species, and 40 other bacterial species as templates in the qPCR. Quantification of the target species and total *Pseudomonas* counts resulted in linear detection ranges of approx. 10^3–10^7 cfu/ml, which correspond well to common *Pseudomonas* counts in raw milk. Application of the assay using 60 raw milk samples from different dairies showed good agreement of total *Pseudomonas* counts calculated by qPCR with cell counts derived from cultivation. Furthermore, a remarkably high variability regarding the species composition was observed for each milk sample, whereby *P. lundensis* and *P. proteolytica*/*P. gessardii* were the predominant species detected.

Key points
- *Multiplex qPCR for quantification of seven proteolytic Pseudomonas species and total Pseudomonas counts in raw milk*
- *High specificity and sensitivity via hydrolysis probes against aprX and rpoB*
- *Rapid method to determine Pseudomonas contamination in raw milk and predict spoilage potential*

Keywords Multiplex quantitative PCR · *Pseudomonas* · Proteolytic milk spoilage · *aprX*

Introduction

Premature spoilage of ultra-high temperature (UHT) milk and milk products due to microbial extracellular enzymes is challenging for the dairy industry, both from an economic and a technical point of view (Hantsis-Zacharov and Halpern 2007; Marchand et al. 2009b; Stoeckel et al. 2016a, b; von Neubeck et al. 2016). Cold storage of raw milk before processing favors the growth of psychrotolerant bacteria, especially *Pseudomonas*, which soon dominate the microbiota (Lafarge et al. 2004; De Jonghe et al. 2011; von Neubeck et al. 2015). Several *Pseudomonas* species produce the extracellular, caseinolytic peptidase AprX, which is heat stable and remains partly active even after UHT treatment. Residual AprX activity can then cause negative effects in milk, such as off-flavors, particle formation, fat separation, or age gelation, all leading to instability and shelf life reduction of processed dairy products (McKellar 1981; Sørhaug

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and Stepaniak 1997; Matéos et al. 2015; Stoeckel et al. 2016a; Marchand et al. 2017).

The alkaline zinc-metallopeptidase AprX, belonging to the serralysin protease family, has a molecular weight of 45–50 kDa and is encoded by the polycistronic *aprX-lipA2* operon (Schokker and van Boekel 1997; Woods et al. 2001; Marchand et al. 2009b). For many *aprX*-possessing *Pseudomonas* species, this operon additionally includes genes coding for a peptidase inhibitor (AprI), an ABC-transport system (AprDEF), two putative autotransporters (PrtA and PrtB), and a lipase (LipA) (Duong et al. 2001; Woods et al. 2001; Maier et al. 2020). Several studies revealed a high variability of milk-associated *Pseudomonas* species and strains regarding their proteolytic potential, which has been proposed to be due to different gene expression and regulation mechanisms (Dufour et al. 2008; Marchand et al. 2009b; Bagliniere et al. 2012; von Neubeck et al. 2015; Caldera et al. 2016). However, genetic variations also seem to play a role, as *aprX* gene sequences of *Pseudomonas* spp. isolated from raw milk were shown to be very heterogeneous (Marchand et al. 2009b). Moreover, different *aprX-lipA2* operon structures were identified in the genus and a correlation between the type of operon organization and the proteolytic potential of pseudomonads was observed (Maier et al. 2020). Regarding the occurrence in raw milk, *Pseudomonas proteolytica*, *Pseudomonas lundensis*, *Pseudomonas lactis*, *Pseudomonas fragi*, *Pseudomonas protegens*, *Pseudomonas gessardii*, and *Pseudomonas fluorescens* were found to be the most frequent species, revealing various proteolytic capacities. While strains of *P. proteolytica*, *P. lactis*, *P. protegens*, *P. gessardii*, and *P. fluorescens* exhibited mainly high proteolytic activity, isolates of *P. lundensis* or *P. fragi* had middle or low proteolytic potential (Marchand et al. 2009a; De Jonghe et al. 2011; Baur et al. 2015; von Neubeck et al. 2015; Caldera et al. 2016; Glück et al. 2016; Maier et al. 2020).

Sensitive and rapid applications for determination of milk-spoiling *Pseudomonas* strains or AprX amounts are required to control raw milk quality and avoid deterioration of processed dairy products. Time-consuming culturing on selective media is not suitable to predict the spoilage potential of raw milk samples prior to processing. Regarding molecular methods, only a few immunological assays with monoclonal antibodies directed against single AprX proteins of specific *Pseudomonas* strains have been developed, which are not appropriate for a broader application in raw milk containing multiple species (Birkeland et al. 1985; Clements et al. 1990; Matta et al. 1997). Moreover, PCR-based approaches have been performed using *aprX* as a target gene to indirectly detect the spoilage potential (Martins et al. 2005; Marchand et al. 2009b; Machado et al. 2013). However, these methods were applied in pasteurized, reconstituted, or sterilized milk and are not sensitive enough to be used in raw milk, having a lower detection limit of, e.g., 10^7 colony-forming units (cfu) per ml (Machado et al. 2015). Also, most former molecular assays focused on the *aprX* or peptidase detection of *P. fluorescens* strains, neglecting other common milk-spoiling species, such as members of the *P. gessardii* and *P. fragi* subgroup.

Consequently, until now, there is no genetic method to discriminate between distinct *Pseudomonas* species with various proteolytic activity present in raw milk. Thus, the aim of this study was to develop a species-specific multiplex qPCR assay, able to quantify seven of the most frequent and proteolytic *Pseudomonas* species in raw milk as well as the total *Pseudomonas* counts. Overall, two triplex assays were established using species-specific probes, targeting *aprX* gene sequences, and one universal *rpoB* probe, directed against all members of the genus.

Material and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in the Supplementary Table S1. In total, 75 strains of 57 *Pseudomonas* species and isolates of 40 other bacterial species, belonging to 25 different genera, were chosen. Among them, 61 strains originated from raw milk, 18 from environmental samples, 9 from milk or semi-finished milk products, 8 from water, 8 from soil, 4 from food environments, and 4 from human samples. For cultivation, most bacterial strains were grown under aerobic conditions on TS-agar (Carl Roth GmbH, Karlsruhe, Germany) at 30 °C for 24–96 h. *Bifidobacterium longum* was cultivated under anaerobic conditions at 37 °C on TOS-agar (Merck KGaA, Darmstadt, Germany). Members of *Lactobacillus*, *Leuconostoc*, and *Lactococcus* were grown under anaerobic conditions at 30 °C on APT-agar for 48 h (Merck KGaA, Darmstadt, Germany). Overnight cultures of *Pseudomonas* spp. were performed by inoculating 4 ml tryptic soy broth (TSB, Merck KGaA, Darmstadt, Germany) with cell material from one colony and incubated at 30 °C and 150 rpm for 16 h. Cell counts were determined on TS-agar (total cell count) as well as on selective CFC-agar (*Pseudomonas* cell count, Merck KGaA, Darmstadt, Germany) after incubation at 30 °C for 24 h.

Raw milk samples

For spiking experiments, fresh raw milk was obtained from a test farm of TUM (Forschungsstation Veitshof, Freising, Germany) and stored at − 20 °C until use, to ensure constant experimental conditions. For validation of the qPCR assay, 60 raw milk samples from 13 different dairies located all over Germany were analyzed. All samples were shipped refrigerated for 1–3 days. Total and *Pseudomonas* cell counts of raw

milk samples were determined immediately after receipt, and the remaining samples were stored at − 20 °C until further processing.

Bacterial DNA extraction from raw milk samples

Bacterial DNA was extracted from raw milk samples using the DNeasy® PowerFood® Microbial Kit (Qiagen N.V., Hilden, Germany) combined with an EDTA pre-treatment. In brief, raw milk samples of 7.2 ml (4 × 1.8 ml) were centrifuged (2 min, 16000*g*, room temperature (RT)), and the supernatants and the covering fat layers were removed. The remaining pellets were then resuspended and united in a total of 1 ml ¼ Ringer's solution (Merck KGaA, Darmstadt, Germany). After adding 300 μl EDTA (0.5 M) and 200 μl 1× TE-buffer, samples were incubated (1 min, RT), centrifuged (2 min, 16000*g*, RT), and the supernatants were carefully removed. Bacterial DNA in the remaining pellets was subsequently isolated following the manufacturer's instructions of the kit. DNA was eluted in 50 μl elution buffer and stored at − 20 °C until use.

Reconstruction of *aprX* and *rpoB* single-gene phylogenies

Protein-coding genes of 61 *Pseudomonas* strains were predicted based on NCBI genome assemblies (Supplementary Table S2) using Prodigal v2.6 (Hyatt et al. 2010). *AprX* and *rpoB* gene sequences were extracted from gene predictions by searching for unidirectional best BLASTp v2.2.25+ (Camacho et al. 2009) hits to the NCBI reference sequences with GenBank identifiers AGL85010.1 and KKJ93525.1, respectively. Subsequently, multiple sequence alignments were calculated with ClustalW (Thompson et al. 1994) and used for maximum likelihood phylogenetic tree reconstruction via the MEGAX v10.0.5 software (Kumar et al. 2018) applying the general time reversible (GTR) model under the assumption of rate heterogeneity (+G) and a proportion of invariant sites (+I). To infer branch confidence values, 500 bootstrap replicates were computed for each tree. Finally, both phylogenies were visualized using the interactive Tree Of Life (iTOL) v5.3 online tool (Letunic and Bork 2019). The strain *P. aeruginosa* WS 5022 served as outgroup to root the trees.

Estimation of *aprX* and *rpoB* sequence similarities

Pairwise p-distances of *aprX* and *rpoB* gene sequences from *Pseudomonas* strains were calculated based on single-gene multiple sequence alignments using the MEGAX v10.0.5 software (Kumar et al. 2018). After subtracting distance values from 1 and multiplication by a factor of 100, pairwise sequence similarities were obtained. The outgroup strain *P. aeruginosa* WS 5022 was excluded from the comparison.

Primer and probe design

All primer and hydrolysis probes used in this study and their main characteristics are listed in Table 1. In total, five species-specific hydrolysis probes and primers targeting *aprX* were created to detect the following species: *P. proteolytica*, *P. gessardii* and *P. gessardii*-like species (probe 1; P1); *P. fluorescens, P. lactis* and *P. lactis*-like species (probe 2; P2); *P. protegens* and *P. protegens*-like species (probe 3; P3); *P. fragi* (probe 4; P4); *P. lundensis* and *P. lundensis*-like species (probe 5; P5). Additionally, one universal *rpoB* probe (probe 6; P6) and primer pair, targeting all members of *Pseudomonas*, were produced. For design, *aprX* and *rpoB* sequences of 61 *Pseudomonas* strains (30 type strains and 31 environmental isolates) were selected. All isolates and associated genome accession numbers are listed in the Supplementary Table S2. Respective sequences were aligned applying MEGA X (Kumar et al. 2018) and conserved regions were identified manually and checked for suitability. The formation of self- and cross-dimers of primers and probes was analyzed using Multiple Primer Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts), and hairpin formation was tested via OligoCalc (Kibbe 2007). Resulting primers had an annealing temperature between 55.2 and 58.2 °C, a GC content of 47.6 to 64.7%, and a length of 17 to 21 nucleotides. Hydrolysis probes revealed an annealing temperature between 63.1 and 65.8 °C, a GC content of 56.5 to 68.4%, and a length between 19 and 23 nucleotides. All oligonucleotides and hydrolysis probes were obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany).

qPCR optimization and conditions

Quantitative PCR was performed with the real-time PCR detection system CFX96/C1000 Touch™ (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the CFX Maestro™ software and the following reaction conditions: Initial denaturation step at 95 °C for 2 min and 35 cycles including denaturation at 95 °C for 5 s and annealing/extension at 61 °C for 15 s. Optimal primer concentration was determined separately for each primer pair via a SYBR green qPCR assay. For this, a total reaction volume of 10 μl was used, including 5 μl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 1 μl per primer in different concentrations (200 nM, 400 nM, or 600 nM), and 1 μl target DNA. The optimal quantity of the hydrolysis probes was subsequently determined by applying different probe concentrations (150 nM, 200 nM, 250 nM) with the previously defined primer concentrations in singleplex probe-based qPCR. Singleplex and multiplex probe-based qPCR was performed in 10 μl reaction volume, containing 5 μl of the SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories Inc., Hercules, CA,

Table 1 Composition and characteristics of the two triplex qPCR assays. Assay set 1 targets abundant, high proteolytic *Pseudomonas* species, and set 2 detects abundant, but less proteolytic species and total pseudomonads. Sequences of six primer pairs (Pr_F and Pr_R) and hydrolysis probes (P1–P6), final concentrations, amplicon length, target genes, and target species are listed. Probes' fluorophores (5′-ends) and quenchers (3′-ends) are shown in bold

Primer/probe	Sequence (5′-3′)	Conc. [nM]	Amplicon length [bp]	Target gene	Target species
Assay set 1					
Pr1_ F	GCACCAATGASAAGTACCACA	400	135	*aprX*	*P. proteolytica*, *P. gessardii*, *P. gessardii*-like
Pr1_R	GTATGGCCGATCTCGTGG	600			
P1	**CY5**-CACGGATGGCACCTCGTGGTAC-**BHQ2**	200			
Pr2_F	ACCTTCCTCACCTCGGCT	600	137	*aprX*	*P. fluorescens*, *P. lactis*, *P. lactis*-like
Pr2_R	GGTAAAGGTCACGTTGGCA	600			
P2	**TexasRed**-AACACCCAGCAGAAAGCACA GGC-**BHQ2**	200			
Pr3_F	GCATCTGCCGAACAACAAC	400	85	*aprX*	*P. protegens*, *P. protegens*-like
Pr3_R	CGATCGTATTGGTGGCTGA	200			
P3	**FAM**-CCGCAGCAAGTTCGGCGTATAAC-**BHQ1**	150			
Assay set 2					
Pr4_F	AGCAGCATTGTCCGTTGG	400	130	*aprX*	*P. fragi*
Pr4_R	CGGTGGTGAGCGAAGGT	600			
P4	**FAM**-CGGCAAACACCGGCAGTTCTG-**BHQ1**	200			
Pr5_F	TGCTGGCCTGGTTGTAGC	600	92	*aprX*	*P. lundensis*, *P. lundensis*-like
Pr5_R	TCACCGGGATTACTCATCTCA	600			
P5	**TexasRed**-ACGACCGCATCACCCGCCT-**BHQ2**	200			
Pr6_F	CAGCCGYTGGGTGGTAA	400	130	*rpoB*	*Pseudomonas* spp.
Pr6_R	CCGTTCACATCGTCCGA	200			
P6	**CY5**-AGTTCGGTGGTCAGCGTTTCGG-**BHQ2**	150			

USA), primers, and probe in optimized concentrations (Table 1) and 1 or 2 μl DNA template, depending on the experiment. Multiplex qPCR utilizing six probes was split into two triplex assays, containing each three hydrolysis probes and the respective primer pairs as listed in Table 1.

Production of artificial DNA mixtures

In order to evaluate the multiplex qPCR assays, complex DNA pools were generated to be used as templates. Two strains per target species of each species-specific probe (P1–P5) were selected as representatives. Strains were grown on TS-agar plates for 24 h, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen N.V., Hilden, Germany) according to manufacturer's specifications. Then, tenfold dilution series containing 100 to 0.01 ng/μl DNA of each strain were prepared and concentrations were checked using the Qubit 2.0 Fluorometer (Invitrogen AG, Carlsbad, CA, USA). Subsequently, identical concentrations of five target DNAs, each of which is detected by one of the five species-specific probes, were combined in a single DNA pool. In total, six distinct DNA mixtures (pool 1–6) with various target DNA compositions were prepared, which are summarized in Supplementary Table S3. In addition, each DNA mixture was generated in five different total DNA concentrations (0.01, 0.1, 1.0, 10, and 100 ng/μl) using the dilution series of the single target DNAs. Thus, the final concentration of target DNAs per species-specific probe (P1–P5) was between 0.002 and 20 ng/μl in the DNA pools. With regard to the universal *rpoB* probe (P6), detecting all pseudomonads, target DNA concentration ranged from 0.01 to 100 ng/μl in the DNA mixtures.

Amplification efficiency and sensitivity of the qPCR assays

For each hydrolysis probe, singleplex qPCR was performed before conducting multiplex qPCR, in order to check for probe functionality and possible interfering interactions between primer and probes. For both single- and multiplex qPCR, all six DNA mixtures (Supplementary Table S3) were used in different concentrations as templates. The quantification cycle (C_q) values obtained per probe from differing target DNA concentrations in the singleplex approach were compared with corresponding C_q values received from multiplex qPCR. For the determination of reaction efficiencies, regression lines were created by plotting the C_q values versus the log of the target DNA concentration used for qPCR. The

amplification efficiency (E) was calculated for each probe from the slopes using the formula: $E = 10^{\frac{-1}{\text{slope}}} - 1$

In order to evaluate the sensitivity of the qPCR assay, the linear dynamic range was determined as well as the lower limit, defined as the number of detectable gene copies when applying the minimum target DNA concentration (0.002 ng for probes P1–P5 and 0.01 ng for probe P6). Copy numbers of *aprX* (for P1–P5) and *rpoB* (P6) were calculated for each probe separately, using the following formula (Staroscik 2011-2020):

$$\text{gene copy number} = \frac{\text{amount of genomic DNA [ng]} \times \text{Avogadro constant}\left(6.022 \times 10^{23}\left[\frac{1}{\text{mol}}\right]\right)}{\left(\text{genome size} \times \text{mass of dsDNA}\left(660\left[\frac{\text{g}}{\text{mol}}\right]\right)\right) \times 10^{9}\frac{\text{ng}}{\text{g}}}.$$

As *aprX* and *rpoB* present single-copy genes, the genome number is equivalent to the number of gene copies. Genome sizes of 18 target strains, which were used for the production of the DNA mixtures, were taken from NCBI and averaged per probe: 6265591 bp (P1), 6528297 bp (P2), 6799673 bp (P3), 5227135 bp (P4), 5131361 bp (P5), and 6171092 bp (P6). The average molecular mass per base pair (dsDNA) was defined as 660 g/mol.

Specificity of qPCR assays

In addition to 26 *Pseudomonas* strains, belonging to the seven target species (plus five very closely related species), 49 *Pseudomonas* strains from 45 non-target species and isolates of 40 other bacterial species were selected in order to check the specificity of the qPCR assays (Supplementary Table S1). Selection of strains was based on their relevance in raw milk and their phylogenetic proximity to the target species. Strains were grown on TS-agar plates for 24 h, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen N.V., Hilden, Germany) according to manufacturer's specifications. DNA concentration was then measured via Qubit 2.0 Fluorometer (Invitrogen AG, Carlsbad, CA, USA) and adjusted to 1–2 ng/μl. One microliter DNA was used as template for the two triplex qPCR assays in a 10 μl reaction volume leading to a final DNA concentration of 0.1–0.2 ng/μl.

Generation of standard curves in raw milk

For the correlation of C_q values and cell counts, three to nine target strains were selected per species-specific probe and all of them (in total 26 strains) for the universal probe (Supplementary Table S1 in bold). Strains were grown in TSB at 30 °C and 150 rpm for 16 h, before 2 ml of each overnight culture was centrifuged (1 min, 13000*g*, RT). The supernatant was discarded, and the pellet resuspended in 10 ml of fresh raw milk. Afterward, a fivefold dilution series ($1{:}5^1$ to $1{:}5^9$) of this sample was prepared with fresh raw milk. The dilution steps $1{:}5^2$, $1{:}5^3$, $1{:}5^5$, $1{:}5^7$, and $1{:}5^9$ were selected for cell count determination by plating and DNA extraction using the DNeasy® PowerFood® Microbial Kit (Qiagen N.V., Hilden, Germany) with the above-listed protocol. Extracted, bacterial DNA of each strain was then applied as template (2 μl in 10 μl reaction volume) in the two triplex qPCR assays, and C_q values were determined of all samples in two technical replicates. For the *rpoB* probe (P6), the dilution step $1{:}5^2$ was not considered due to the high amount of target DNA. In parallel, *Pseudomonas* counts of all sample dilutions were quantified on CFC-agar plates. The *Pseudomonas* count of untreated raw milk was determined (1.6×10^3 cfu/ml) and subtracted from the counts of spiked raw milk samples. For standard curves, logarithmic cell counts per milliliter were plotted against the respective C_q values of identical samples.

Results

Setup of multiplex qPCR for species-specific and total *Pseudomonas* detection

Besides the quantification of total pseudomonads, the novel qPCR assay was developed to specifically detect seven milk-relevant *Pseudomonas* species, namely *P. proteolytica*, *P. gessardii*, *P. lactis*, *P. fluorescens*, *P. protegens*, *P. lundensis*, and *P. fragi.* As target genes, *aprX* was chosen for species-specific detection, and the conserved *rpoB* gene for the quantification of total *Pseudomonas* counts.

In order to check the suitability of the selected target genes and to determine the number of probes and primers needed, phylogenetic analyses using *aprX* and *rpoB* sequences of 61 *Pseudomonas* strains, assigned to 46 different species, were performed. The overall topology of the phylogenetic *aprX* tree was similar to the one based on *rpoB* sequences (Fig. 1 and Supplementary Fig. S1), considering the classification of strains into the previously described *Pseudomonas* subgroups (Gomila et al. 2015; Peix et al. 2018; Maier et al. 2020) and the distribution of species within these groups. However, the *aprX* sequences were more variable and discriminative (66.0–100.0% sequence similarity range), thus enabling a higher resolution than the conserved *rpoB* gene sequences (85.1–100.0% sequence similarity range), which served for the design of a genus-specific probe and respective primers. With respect to the seven chosen target species, the *aprX* tree exhibited a distribution of the 18 representative strains in four *Pseudomonas* subgroups, namely *P. fluorescens*, *P. gessardii*, *P. fragi*, and *P. chlororaphis*. Sequences of isolates from *P. lactis* and *P. fluorescens*, both belonging to the *P. fluorescens* subgroup, as well as the ones from *P. proteolytica* and *P. gessardii*, being part of the *P. gessardii* subgroup, showed a very high inter-species

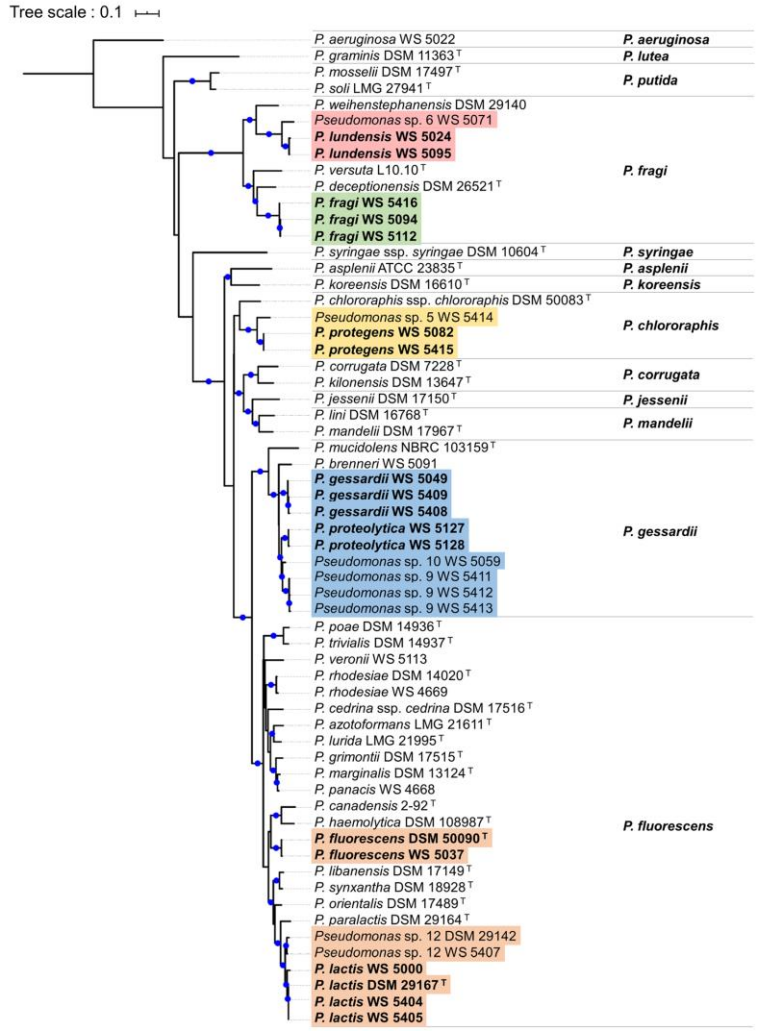

Fig. 1 Maximum likelihood phylogeny of the *aprX* gene based on 1,296 positions in the multiple sequence alignment of 61 distinct *Pseudomonas* strains. Molecular evolution was inferred by the GTR+G+I model, and the tree was outgroup-rooted (*P. aeruginosa* WS 5022). Branches with high bootstrap support (≥ 70% of 500 replicates) are marked with blue circles. Target strains of the species-specific probes are highlighted in blue (P1), orange (P2), brown (P3), green (P4), and red (P5). Strains were assigned to 13 monophyletic groups, whose names are listed in bold to the right of the tree

sequence similarity of at least 91.9 and 94.1%, respectively. For these very closely related species, the design of a single hydrolysis probe and primer pair, targeting the *aprX* sequences from members of both species, was possible. In contrast, the *aprX* sequences of *P. lundensis* and *P. fragi* strains, all belonging to the *P. fragi* subgroup, differed largely (maximum inter-species sequence similarity of 81.1%), and therefore, separate probes and primers were created for each species. Also, for *P. protegens* strains, being located in the *P. chlororaphis* subgroup, the design of an additional probe plus primers was necessary. Consequently, a total of five hydrolysis probes (P1–P5) and primer pairs were generated to detect all seven target species. Besides the 18 strains of the target species, eight very closely related isolates were taken into account for probe and primer design (Supplementary Table S2), as they were shown to be frequently present in raw milk and exhibit comparable proteolytic characteristics (von Neubeck et al. 2015; Maier et al. 2020). According to our phylogenomic study, these strains do not belong to species with validly described names (Maier et al. 2020) and will be

referred to as "*P. lactis*-like," "*P. gessardii*-like," "*P. lundensis*-like," and "*P. protegens*-like" in the following.

The six hydrolysis probes and respective primer pairs were split into two triplex qPCR reactions, whose compositions are summarized in Table 1. Assay 1 comprised three probes (P1–P3) and primer pairs to quantify common and highly proteolytic species, namely *P. proteolytica*, *P. gessardii* and *P. gessardii-like* species (P1); *P. fluorescens*, *P. lactis* and *P. lactis*-like species (P2); as well as *P. protegens* and *P. protegens*-like species (P3). Assay 2 contained two probes (P4 and P5) and respective primers detecting *P. fragi* (P4), and *P. lundensis* plus *P. lundensis*-like species (P5), which are less proteolytic, but abundant in raw milk samples. Moreover, assay 2 was complemented with the universal *Pseudomonas* primers and probe P6 for quantification of total *Pseudomonas* counts. For all designed primers and probes, the optimal concentrations were specified separately by singleplex qPCR (Table 1), and an optimal annealing temperature of 61 °C was determined by gradient qPCR (data not shown).

Efficiency and linearity of single- and multiplex qPCR

For determination of the amplification efficiencies, six defined DNA pools of target and non-target DNA were produced (Supplementary Table S3) and various dilutions thereof were applied as templates in qPCR. After testing each probe-primer combination separately in a singleplex assay, three probes plus primers were combined in the triplex approach. Therefore, DNA of two to six different target strains was applied for each of the five species-specific probes (P1–P5), and DNA of all 18 target strains was employed for the universal *Pseudomonas* probe (P6). Averaged C_q values were calculated for each hydrolysis probe with its primer pair (Table 2). Thereby, linear correlations between C_q values and DNA concentrations were observed for all six probes in singleplex and multiplex qPCR, yielding high correlation coefficient (R^2) values of > 0.975 and PCR amplification efficiencies (E) of 85–97%. Since mean C_q values of singleplex and multiplex qPCRs were highly comparable (Table 2), possible interactions between the different probes and primer pairs in the multiplex reactions do not adversely affect target detection or amplification.

For the five species-specific probes (P1–P5), target DNA amounts from 2 to 0.0002 ng/μl (final concentrations in 10 μl reaction volume) and for the universal *Pseudomonas* probe P6 from 10 to 0.001 ng/μl were detectable, demonstrating a wide linear dynamic range over 4 log-steps. For qPCR with P1–P5, calculated minimal *aprX* gene copy numbers lay between 268 and 356. Using the universal *Pseudomonas* probe (P6), a minimum of approx. 1.5×10^3 *rpoB* gene copies was detectable with 0.001 ng/μl target DNA. Since no greater C_q value than 32.5 was received for all probes when applying the lowest target DNA amounts, a cut-off value of 33 was defined for further experiments, and higher C_q values were considered unquantifiable.

Specificity of the multiplex qPCR assay

To verify the specificity of the assay, 1–2 ng/μl genomic DNA of 75 *Pseudomonas* strains (target and non-target strains), assigned to 57 different species, and of 40 other bacterial species was applied as template in the qPCR assays. For strain selection, *Pseudomonas* isolates of the target species and their closest relatives, as well as representatives of the whole genus, were considered. Other bacterial species were chosen due to their phylogenetic proximity to the genus *Pseudomonas* and/or their relevance in milk and milk products.

Using the five species-specific probes (P1–P5) targeting *aprX*, all 18 strains of the defined seven target species were detected successfully, yielding C_q values from 18.85 to 22.13 (Table 3). Moreover, the eight very closely related isolates, which belong to *P. gessardii*-like, *P. lactis*-like, *P. lundensis*-like, and *P. protegens*-like species, resulted in positive signals in the same range (C_q 19.59–21.27).

For the 50 non-target pseudomonads tested, no false-positive signals were received using P3, P4, and P5, demonstrating a very high specificity. However, P1 and P2, detecting multiple target species at once, showed few false-positive results (Supplementary Table S4). In case of P1, a very weak signal was measured with DNA of *P. marginalis* DSM 17967 (C_q 32.1). Regarding P2, detecting *P. lactis* and *P. fluorescens*, false-positive signals were obtained for four strains of the closely related species *Pseudomonas haemolytica*, *Pseudomonas paralactis*, *Pseudomonas orientalis*, and *Pseudomonas synxantha* (C_q 24.4–27.6). For the 40 other bacterial species tested, no cross-reactivity was observed using P1–P5, except a negligible signal for *Streptococcus pyogenes* DSM 2071 (C_q 32.91) (Supplementary Table S4), underlining the high specificity of the designed primers and probes.

Via the universal *rpoB* probe (P6), 74 out of 75 *Pseudomonas* strains tested were successfully detected, generating C_q values between 17.45 and 22.69 (Supplementary Table 3 and Supplementary Table S4). Only the signal received from DNA of *Pseudomonas stutzeri* WS 5018 was considerably weaker (C_q 27.13). When testing the 40 other bacterial species, very weak unspecific signals were obtained for 5 isolates with P6 (Supplementary Table S4). Among them, *Pseudoalteromonas haloplanktis* WS 5482 yielded the highest signal with a C_q of 29.61, while the others showed even higher C_q values ranging from 30.96 to 32.91.

Quantification of *Pseudomonas* via multiplex qPCR

In order to generate standard curves for quantification of cells, C_q values from multiplex qPCR were correlated with the

Table 2 Correlation between C_q values and DNA concentration using the six *Pseudomonas* probes (P1–P6) in singleplex and multiplex qPCR. As templates, serial dilutions of six artificial DNA pools (1–6) were applied, each containing DNA of five different *Pseudomonas* strains in equal quantities (Supplementary Table S3). In total, DNA of six different target strains (from pool 1 to 6) was measured for the species-specific probes P1 and P2, and DNA of each two strains (from pool 1 and 2) for probes P3, P4, and P5. For the universal *Pseudomonas* probe P6, all 18 strains from the six DNA pools were taken into account. All measurements were conducted in two technical replicates, and averaged C_q values, amplification efficiency, and coefficients of determination are shown

Probe	Template DNA from	Concentration of target DNA [ng/µl]	Singleplex qPCR		Multiplex qPCR	
			Mean C_q value	Efficiency / R^2 value	Mean C_q value	Efficiency / R^2 value
P1	6 target strains (pool 1–6)	2	17.22 ± 0.63	87%	16.06 ± 0.58	85%
		0.2	20.44 ± 0.52	0.979	19.46 ± 0.40	0.981
		0.02	24.18 ± 0.55		23.25 ± 0.60	
		0.002	28.24 ± 0.60		27.30 ± 0.64	
		0.0002	32.44 ± 1.28		31.65 ± 1.35	
P2	6 target strains (pool 1–6)	2	15.72 ± 0.69	89%	15.49 ± 0.21	90%
		0.2	19.50 ± 0.15	0.990	19.12 ± 0.51	0.992
		0.02	23.02 ± 0.15		22.57 ± 0.42	
		0.002	26.65 ± 0.36		26.29 ± 0.23	
		0.0002	30.72 ± 0.91		30.25 ± 0.39	
P3	2 target strains (pool 1 + 2)	2	16.77 ± 0.16	90%	16.04 ± 0.25	88%
		0.2	20.21 ± 0.24	0.998	19.39 ± 0.13	0.995
		0.02	23.92 ± 0.25		22.90 ± 0.24	
		0.002	27.94 ± 0.25		27.03 ± 0.11	
		0.0002	32.37 ± 0.31		31.25 ± 0.07	
P4	2 target strains (pool 1 + 2)	2	16.20 ± 0.06	92%	14,70 ± 0.20	93%
		0.2	19.52 ± 0.20	0.999	17,88 ± 0.30	0.993
		0.02	23.03 ± 0.07		21,31 ± 0.15	
		0.002	27.01 ± 0.08		25,26 ± 0.08	
		0.0002	31.37 ± 0.55		29,74 ± 0.43	
P5	2 target strains (pool 1 + 2)	2	15.50 ± 0.09	94%	14.99 ± 0.14	97%
		0.2	18.96 ± 0.07	0.998	18.28 ± 0.12	0.998
		0.02	22.29 ± 0.06		21.58 ± 0.16	
		0.002	26.10 ± 0.19		25.21 ± 0.16	
		0.0002	30.71 ± 0.51		29.80 ± 0.56	
P6	18 target strains (pool 1–6)	10	13.91 ± 0.44	93%	13.20 ± 0.11	95%
		1	17.48 ± 0.38	0.989	16.43 ± 0.11	0.998
		0.1	20.87 ± 0.44		19.90 ± 0.21	
		0.01	24.41 ± 0.38		23.54 ± 0.14	
		0.001	28.42 ± 0.46		28.2 3 ± 0.51	

corresponding *Pseudomonas* counts from cultivation. Therefore, 26 *Pseudomonas* target strains were chosen (Supplementary Table S1), and cells were serially diluted in fresh raw milk. From selected dilution steps, cell counts were determined by cultivation, and in parallel, DNA was extracted and used as template for the two triplex qPCR assays.

Linear correlation between *Pseudomonas* cell counts and C_q values was obtained for each of the six hydrolysis probes, revealing C_q values from 11.9 to 33.0 (Fig. 2). In agreement with the previously defined cut-off value, C_q values exceeding 33 were not detected. All standard curves yielded good efficiencies of 78–88% and high R^2 values of 0.944 to 0.986. However, the R^2 values of the probes P1, P2, and P6, detecting multiple species, were slightly lower than the ones from P3, P4, and P5, each targeting only a single species (Fig. 2, Table 3). The dynamic range of detection was found to be linear between ~ 10^3 and 10^7 cfu/ml for all probes, covering common *Pseudomonas* cell counts in raw milk. The lowest quantifiable cell amounts were also theoretically calculated for the stated C_q threshold of 33 via the standard curves, resulting in cell counts between 2 and 9 × 10^2 cfu/ml (P1: 561 cfu/ml; P2: 262 cfu/ml; P3: 556 cfu/ml; P4: 490 cfu/ml; P5: 359 cfu/ml; P6: 869 cfu/ml).

Table 3 C_q values from triplex qPCR assays, applying five species-specific probes (P1–P5) and the universal *Pseudomonas* probe (P6). Genomic DNA from 26 target strains was used as template in a final concentration of 0.1–0.2 ng/µl. Mean values of two technical replicates per measurement are shown. Hyphens (-) represent no signal in qPCR. C_q values above the defined threshold of 33 were considered as not quantifiable and are given in brackets

	Species-specific hydrolysis probes					Universal *Pseudomonas probe*
Target Strains (P1–P5)	P1	P2	P3	P4	P5	P6
Pseudomonas gessardii WS 5049^{P1}	21.05	-	-	-	-	20.70
Pseudomonas gessardii WS 5408^{P1}	21.05	-	-	-	-	19.52
Pseudomonas gessardii WS 5409^{P1}	21.02	-	-	-	-	19.73
Pseudomonas proteolytica WS 5127^{P1}	20.08	-	-	-	-	20.51
Pseudomonas proteolytica WS 5128^{P1}	19.68	-	-	-	-	20.16
Pseudomonas sp. 10 WS 5059^{P1}	19.59	-	-	-	-	19.89
Pseudomonas sp. 9 WS 5411^{P1}	20.46	-	-	-	-	20.41
Pseudomonas sp. 9 WS 5412^{P1}	20.86	-	-	-	-	19.95
Pseudomonas sp. 9 WS 5413^{P1}	20.67	-	-	(34.81)	-	20.51
Pseudomonas fluorescens DSM 50090^{T,P2}	-	22.13	-	-	-	20.83
Pseudomonas fluorescens WS 5037^{P2}	-	20.32	-	-	-	20.00
Pseudomonas lactis DSM 29167^{T,P2}	-	20.64	-	-	-	19.48
Pseudomonas lactis WS 5000^{P2}	-	20.03	-	-	-	19.06
Pseudomonas lactis WS 5404^{P2}	-	21.86	-	-	-	20.39
Pseudomonas lactis WS 5405^{P2}	-	21.72	-	-	-	19.73
Pseudomonas sp. 12 DSM 29142^{P2}	-	21.05	-	-	-	20.61
Pseudomonas sp. 12 WS 5407^{P2}	-	21.27	-	-	-	20.53
Pseudomonas protegens WS 5082^{P3}	-	-	21.59	-	-	20.25
Pseudomonas protegens WS 5415^{P3}	-	-	20.56	-	-	19.14
Pseudomonas sp. 5 WS 5414^{P3}	-	-	20.48	-	-	19.12
Pseudomonas fragi WS 5094^{P4}	(33.26)	-	-	19.35	-	19.00
Pseudomonas fragi WS 5112^{P4}	(33.46)	-	-	18.85	-	18.53
Pseudomonas fragi WS 5416^{P4}	-	-	-	-	-	19.13
Pseudomonas lundensis WS 5024^{P5}	-	-	-	-	18.57	19.00
Pseudomonas lundensis WS 5095^{P5}	-	-	-	-	18.86	19.29
Pseudomonas sp. 6 WS 5071^{P5}	(33.47)	-	-	-	20.00	18.44

^{P1,P2,P3,P4,P5}: target strain of probe P1–P5. ^{T}: type strain

Application of the qPCR assay to industrial raw milk samples

For assay validation, 60 independent raw milk samples from 13 different German dairies were analyzed. Determination of total and *Pseudomonas* cell counts by cultivation on TSA and CFC-agar, respectively, revealed great differences regarding the relative *Pseudomonas* amounts of samples (Supplementary Table S5).

For qPCR, bacterial DNA was isolated from raw milk samples and used as template in the two triplex assays. Based on the C_q values obtained, corresponding cell counts were calculated using the respective standard curve. Total *Pseudomonas* cell counts determined by qPCR via the universal *Pseudomonas* probe P6 ranged from 8.8×10^2 to 1.2×10^7 cfu/ml (Fig. 3). In two-thirds of the milk samples, cell counts from qPCR and from the cultivation approach did not differ more than 0.5 log, demonstrating a high concordance of the results. Almost one-third of samples showed a difference in cell amounts between 0.5 and 1 log, and only 2 from the 60 samples (no. 29 and 31) varied slightly more than 1 log (Fig. 3).

Regarding the species composition, target species of the five species-specific probes (P1–P5) were detected by qPCRs in all but three raw milk samples tested, while non-target pseudomonads were detected in 53% of the samples (Fig. 4). Remarkably, the occurrence and proportion of each target species differed strongly among the raw milk samples. For each of the five species-specific probes, at least one milk sample contained exclusively the respective target species,

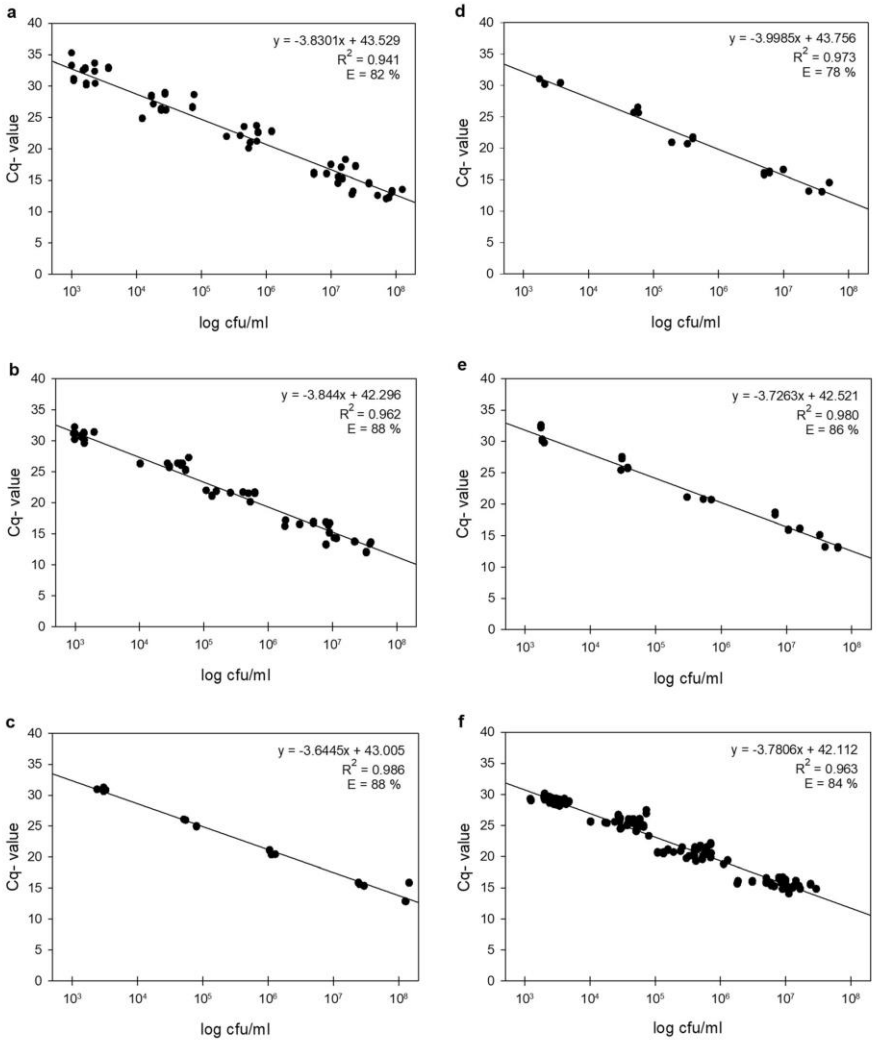

Fig. 2 Standard curves of C_q values from multiplex qPCR using the six *Pseudomonas* probes P1–P6 (**a**–**f**), correlated with cell counts of artificially spiked raw milk samples. 26 representative target strains (9 strains for P1; 8 for P2; 3 for P3, P4 and P5; 26 for P6) were chosen, and cells were serially diluted in fresh raw milk ($1{:}5^2$–$1{:}5^9$). Cell counts of diluted samples were determined by cultivation, and in parallel, DNA of the milk samples was isolated and applied as templates in the two triplex qPCR assays. Averaged C_q values from two technical replicates of each strain were plotted against the respective *Pseudomonas* counts given in log cfu/ml. Regression equation, coefficient of determination (R^2), and amplification efficiency (E) are given for each standard curve

confirming the usefulness of the chosen targets (Fig. 4). In terms of frequency and distribution, the species *P. lundensis* and/or *P. lundensis*-like (targeted by P5) were the most common species, being present in 80% of the milk samples and constituting the largest proportion of the *Pseudomonas* population in 27% of the milk samples. The target species of P1, namely *P. proteolytica*, *P. gessardii* and/or *P. gessardii*-like, were similarly frequent, being identified in 73% of the samples and predominant in 23% of the samples. The target species of P2 (*P. fluorescens*, *P. lactis* and/or *P. lactis*-like),

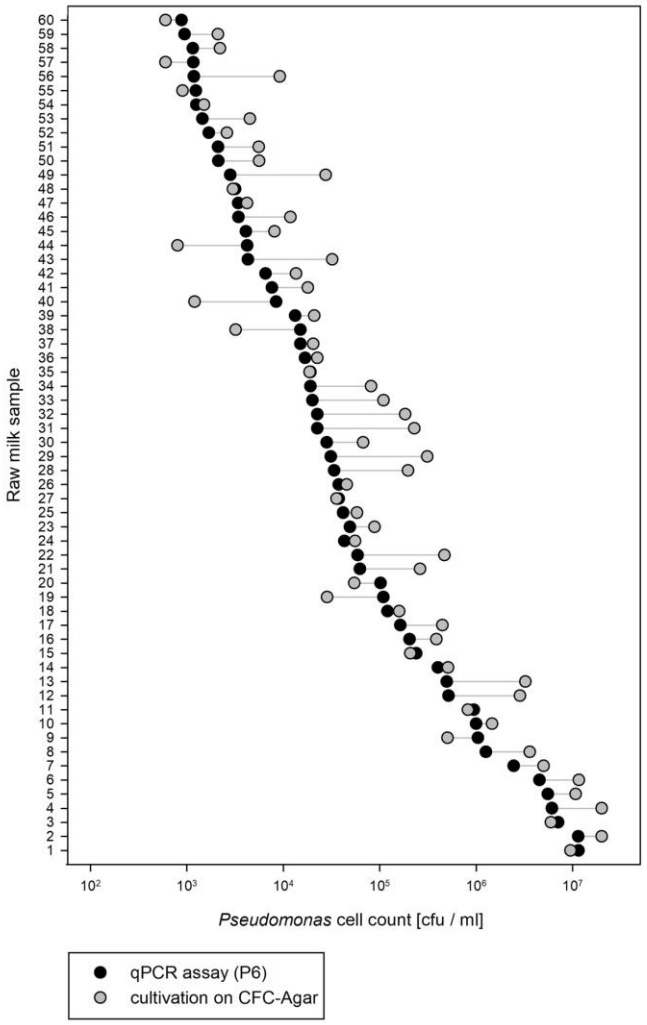

Fig. 3 Comparison of *Pseudomonas* cell counts in 60 raw milk samples, determined by multiplex qPCR assay (black dots) and cultivation on CFC-agar (grey dots). For qPCR, DNA was isolated from each raw milk and applied as template in the assay using the universal *Pseudomonas* probe P6. Cell counts were calculated from received C_q values by linear regression analysis of a standard curve. Deviations between cell counts obtained with multiplex qPCR assay and plating are indicated by grey lines

P4 (*P. fragi*), and P3 (*P. protegens* and/or *P. protegens*-like) were detected in 60%, 42%, and 7% of the milk samples and accounted for the major share of pseudomonads in only 7%, 8%, and 2% of the samples, respectively. Finally, non-target pseudomonads presented the largest proportion in 33% of the raw milk samples (Fig. 4).

In general, no correlation between total *Pseudomonas* cell counts and distribution of certain target species was observed in the analyzed raw milk samples; however, samples with *Pseudomonas* counts < 10^4 cfu/ml tended to comprise more different species than samples with higher cell counts.

Discussion

For the food industry, sensitive and rapid detection methods are crucial to perform a risk assessment and ensure the safety and quality of its products. In recent years, the development of multiplex qPCR assays to detect specific microorganisms in various food matrices has increased rapidly. Utilizing several probes with diverse fluorophores attached, multiplex qPCR enables the co-amplification and differentiation of multiple targets in a single reaction, presenting a cost- and time-saving alternative to singleplex qPCR or cultivation-dependent methods. So far, the

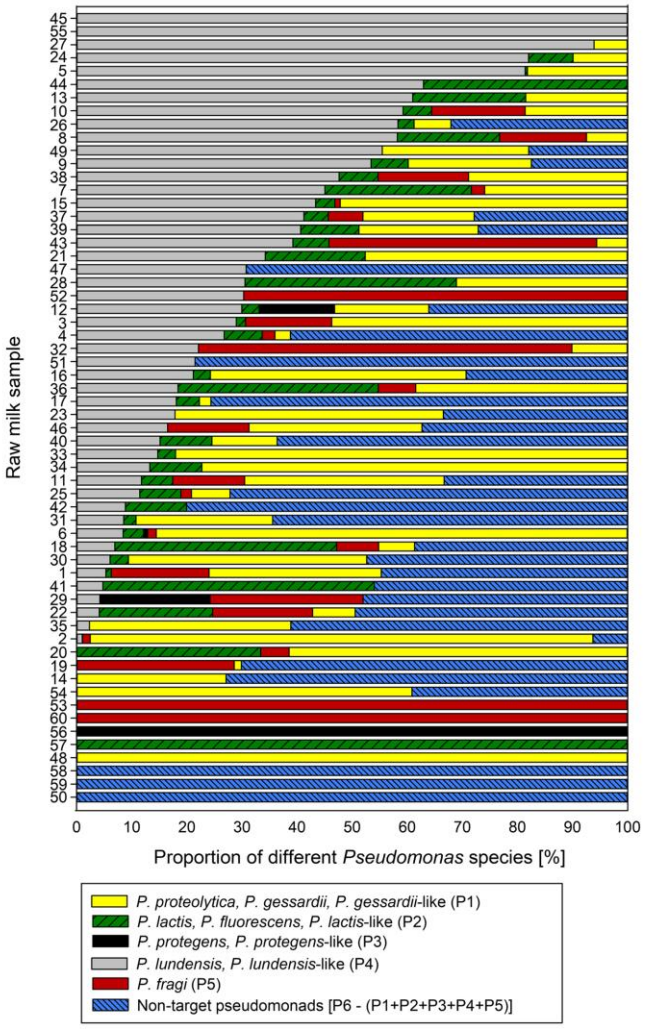

Fig. 4 Distribution of different target species and non-target pseudomonads in 60 raw milk samples, determined by qPCR using five species-specific probes (P1–P5) and one universal *Pseudomonas* probe (P6). Cell counts of the target species and total *Pseudomonas* counts were calculated for each sample via standard curves and C_q values of the respective probes. Results from P6, representing the total count, were defined as 100%, and proportions of target strains accordingly determined. The proportion of non-target pseudomonads was defined by subtracting the sum of all target species (P1–P5) from the total *Pseudomonas* counts (P6)

majority of these applications allow the identification of foodborne pathogens. For example, multiplex qPCR assays have been developed for the detection of *Salmonella* spp., *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter* spp., or *Vibrio* spp. in various foods (Hong et al. 2007; Tebbs et al. 2011; Forghani et al. 2016; Liu et al. 2017; Heymans et al. 2018; Parichehr et al. 2019). In addition, several applications deal with probiotic or beneficial organisms, such as yeasts, *Acetobacter* spp., or different lactic acid bacteria (LAB) in kefir or starter cultures in cheese production (Bottari et al. 2013; Nejati et al. 2020). Moreover, the detection of food-spoiling bacteria, e.g., *Clostridium* spp. in milk and meats or *Bacillus* spp. and *Paenibacillus* spp. in potato salad and milk, has been carried out by multiplex qPCR assays in previous studies (Morandi et al. 2015; Dorn-In et al. 2018; Nakanojp 2020).

For pseudomonads, two non-quantitative multiplex PCR approaches have been performed for different *Pseudomonas* species in meat (Ercolini et al. 2007) and of *P. fluorescens* strains with a biofilm-forming ability (Xu et al. 2017). However, until now, no qPCR assay has been developed for the simultaneous quantification of various milk-spoiling *Pseudomonas* species in

raw milk, which would be very useful for quality assessment in the milk industry. The two triplex assays of this study resulted in the successful enumeration of total *Pseudomonas* counts as well as seven prevalent *Pseudomonas* species in raw milk, enabling discrimination of high and low peptidase producers. Regarding sensitivity, the assays exhibited a linear detection range of approx. 10^3–10^7 cfu/ml with lowest quantifiable cell numbers of 2×10^2–2×10^3 cfu/ml, depending on the TaqMan probe. These results are similar to the detection or quantification limits of other developed qPCR assays enumerating bacteria in spiked milk samples, e.g., *Paenibacillus* spp. and *Bacillus* spp. (Nakanojp 2020); *E. coli* and *Salmonella* spp. (Zhou et al. 2017); and *S. aureus*, *L. monocytogenes*, and *Salmonella* spp. (Ding et al. 2017). For some qPCR assays identifying foodborne pathogens in dairy products, lower detection limits of $< 10^2$ cfu/ml are required and obtained mostly via time-consuming enrichment steps or other sample pre-treatments (Forghani et al. 2016; Heymans et al. 2018; Parichehr et al. 2019). However, as previous studies revealed that average *Pseudomonas* cell counts in raw milk range from 10^2 to 10^5 cfu/ml (Leriche and Fayolle 2012; von Neubeck et al. 2015; Skeie et al. 2019), a higher sensitivity regarding the detection limit of our assay is neither necessary nor beneficial for its application.

When tested using 115 target- and non-target strains, our qPCR assay showed a high level of specificity. Only few false-positive signals were obtained for two out of the five species-specific hydrolysis probes, namely for the multi-target P1 and P2. For P2, this can be explained due to the close phylogenetic proximity of the target species *P. lactis* and *P. fluorescens* to the isolates causing false-positive signals (*P. haemolytica* DSM 108987, *P. paralactis* DSM 29164, *P. orientalis* DSM 17489, and *P. synxantha* DSM 18928). As all these strains were shown to be proteolytic, though less abundant in raw milk (von Neubeck et al. 2015; Maier et al. 2020), the signals are negligible or may even contribute to the detection of proteolytic pseudomonads. Moreover, the universal *Pseudomonas* probe (P6) was shown to be highly specific, detecting all 75 tested *Pseudomonas* strains. Among them, all tested isolates from the 15 species that were previously defined as milk relevant were found (von Neubeck et al. 2015; Caldera et al. 2016; Maier et al. 2020). When 40 non-pseudomonads were tested, the universal probe resulted in five very weak false-positive signals, the highest from DNA of *P. haloplanktis* WS 5482 (C_q 29.6). This psychrophilic marine bacterium has occasionally been isolated from cheese rind, but plays no role in the microbiota of raw milk (Feurer et al. 2004; Quigley et al. 2011; Almeida et al. 2014).

With respect to the enumeration of total *Pseudomonas* counts using P6, the results were in good agreement with cell counts received from cultivation. For the majority of samples, *Pseudomonas* cell counts quantified on selective agar were slightly higher than calculated cell counts via qPCR. This could be due to the growth of some members of *Enterobacteriaceae* or *Acinetobacter* on CFC-agar (Flint and Hartley 1996; Salvat et al. 1997), which are known to be frequently present in raw milk (Hantsis-Zacharov and Halpern 2007; Baur et al. 2015; von Neubeck et al. 2015; Li et al. 2018; Ribeiro Junior et al. 2018; Breitenwieser et al. 2020). Therefore, the determination of total *Pseudomonas* by our qPCR assay presents a highly specific and faster (3 h versus 2–3 days) alternative to the quantification of total counts by cultivation.

Remarkably, when 60 independent raw milk samples from 13 different dairies were analyzed for assay validation, unique compositions of the seven target species and non-target pseudomonads were detected for all samples. Thereby, *P. lundensis* and *P. lundensis*-like species (P5) were found most frequently (in 80% of the samples), closely followed by *P. proteolytica*, *P. gessardii* and *P. gessardii*-like species (P1). Members of *P. lactis* and *P. fluorescens* (P2) and *P. fragi* (P4) were also rather common (present in 60% and 42% of samples, respectively), while strains of *P. protegens* (P3) were relatively rare. Previous studies identifying the *Pseudomonas* population of raw milk or dairy products revealed the same predominant species, namely *P. lundensis*, *P. proteolytica*, *P. gessardii*, *P. fragi*, and *P. fluorescens*. In contrast, representatives of *P. protegens* were less common (Marchand et al. 2009a; De Jonghe et al. 2011; von Neubeck et al. 2015; Caldera et al. 2016). Besides, in all of these studies, other isolates from partly unclassified *Pseudomonas* species were identified, which is also consistent with our results revealing the presence of non-target species in about half of the samples tested.

Since it was shown that the composition of the *Pseudomonas* population varies greatly in raw milk samples, the proportions of highly, middle, and low proteolytic isolates are strongly different, too. Here, our two triplex qPCR assays offer a very useful tool to quantify and simultaneously distinguish between the most common raw milk species, possessing different proteolytic potentials. As triplex assay 1 detects specifically highly proteolytic *Pseudomonas* species (e.g., *P. proteolytica*, *P. gessardii*, or *P. lactis*) and assay 2 species with weaker peptidase activities (e.g., *P. fragi* and *P. lundensis*), they are well suited to estimate the spoilage potential of raw milk. However, for a more accurate risk assessment, future work is needed in order to determine the exact peptidase concentrations causing negative effects in milk. A previous study revealed product defects of UHT milk that was produced from raw milk contaminated with different *Pseudomonas* species, at peptidase activities of ≥ 0.03 pkat/ml (Stoeckel et al. 2016a). Correlations between AprX amounts and the required cell numbers of high as well as of low proteolytic *Pseudomonas* species are necessary for an informed definition of threshold CFU values, which indicate the probability of product spoilage.

In summary, the novel multiplex qPCR assay provides an accurate and rapid technique to quantify the total *Pseudomonas* counts in raw milk and to distinguish between

the most prevalent *Pseudomonas* species with different proteolytic potentials. Thereby, it presents a powerful tool for the dairy industry to predict the spoilage risk and shelf life of raw milk samples at an early stage in order to decide on further processing, e.g., towards UHT or fresh milk products.

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Data availability All data generated during this study are included in the paper or in the electronic supplementary material. Strains and additional raw data are available from the authors upon request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no conflict of interest.

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4 Chapter 3: "Regulatory effects of *aprX-lipA2* operon genes, temperature and growth phase on AprX production in *Pseudomonas proteolytica* WS 5128"

Abstract

Most raw milk-borne *Pseudomonas* spp. secrete a single heat-resistant peptidase, namely AprX, which can lead to quality defects of UHT milk or other milk products with long shelf life. Multiple factors, including growth conditions and the composition of the *aprX-lipA2* operon, have been shown to influence the AprX production of *Pseudomonas* species, leading to diverse and partly strain-specific proteolytic potentials. However, the underlying regulatory mechanisms are largely unknown.

For this purpose, the proteolytic strain *Pseudomonas proteolytica* WS 5128 and six constructed deletion mutants (*P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*aprX* Δ*prtAB, P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*) were investigated regarding the transcription levels of five *aprX-lipA2* operon genes (*aprX*, *aprD*, *prtA*, *prtB* and *lipA2*), the AprX protein amounts, and the proteolytic activities at different growth temperatures (12 °C and 30°C).

Our results indicate that the expression of the *aprX-lipA2* operon in *P. proteolytica* WS 5128 is regulated by temperature on the transcriptional level, as 12 °C-samples exhibited elevated mRNA levels of the operon genes (*aprX*, *aprD*, *prtA* and *prtB*) and higher AprX amounts compared to the 30 °C- samples. Moreover, proteolytic activity and AprX peptidase amounts were initially detected in early stationary phase, even though *aprX* transcripts were already determined in exponential phase. Consequently, the AprX biosynthesis of *P. proteolytica* WS 5128 seems to be modulated translational or post-translational by an unknown signal associated with high cell densities. Also, we obtained first indications of an influence of the *aprX-lipA2* operon genes *prtA*, *prtB,* and *lipA2* on *aprX* expression. Since *P. proteolytica* Δ*prtAB* showed wild type *aprX* transcript levels, but a reduced proteolytic activity and lower AprX amounts than the wild type at 12 °C, an influence of the putative autotransporters PrtA and PrtB on AprX production at the translational level seems possible. However, as this was not observed for 30°C samples and as the single deletion mutants (*P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB)* behaved differently than the double mutant strain, further analyses are required to elucidate the function of PrtA and PrtB. Beyond that, *P. proteolytica* Δ*lipA2* revealed increased *aprX* transcripts compared to the wild type at

30°C, but not at 12 °C, making an influence of the lipase gene *lipA2* on the mRNA stability likely.

In conclusion, *aprX* expression was shown to be regulated by temperature on a transcriptional level and by a yet unknown signal associated with the stationary growth phase on the translational level. Additionally, the operon genes *prtA*, *prtB* and *lipA2* themselves seem to influence AprX production at different regulatory levels, confirming the existence of a complex regulatory network that controls proteolytic activity by AprX in *Pseudomonas* species.

Introduction

Pseudomonas spp. possess an enormous metabolic variability to adapt even to harsh environments. Through the secretion of secondary metabolites and extracellular enzymes, they suppress competitors' growth or provide nutrients for their cell development (Loper et al., 2012, Shahid et al., 2018). As the production of such compounds is very energy-intensive, they usually underlie complex regulation mechanisms. For example, to respond to different environmental stimuli, signals are often perceived directly or indirectly through two-component systems, like the global regulator GacA/GacS, modulating downstream gene expression (Heeb et al., 2002, Haas and Keel, 2003). One extracellular enzyme regulated by the GacA/GacS system is the heat-stable peptidase AprX (Zuber et al., 2003), which is associated with casein degradation in temperature-treated milk products (Stoeckel et al., 2016). AprX is characterized by a rather variable proteolytic activity which is species- and in parts, strain-specific (Baur et al., 2015a). However, as environmental factors such as temperature (Burger et al., 2000), iron (Woods et al., 2001), or calcium (Ertan et al., 2015) also affect the caseinolytic activity, a far more complex regulation is indicated.

Raw milk-associated *Pseudomonas* species were shown to secrete a sole peptidase, the metallopeptidase AprX (Woods et al., 2001, Maier et al., 2020). AprX is sized between 45 and 50 kDa (Marchand et al., 2009), functional at a light acidic to alkaline pH, and presents optimal substrate turnover rates between 37 – 45 °C (Dufour et al., 2008, Glück et al., 2016, Volk et al., 2019). Moreover, the divalent metal ions Zn^{2+} and Ca^{2+} are essential to ensure functionality and stability of AprX (Schokker and van Boekel, 1997). Volk et al. distinguish between two different AprX homologs sharing around 68 % amino acid sequence similarity. It was shown that peptidases of group 2 (present in, e.g., *Pseudomonas proteolytica*, *Pseudomonas lactis*) exhibited 3-30-

times increased substrate turnover rates compared to proteins of group 1 from *Pseudomonas lundensis* or *Pseudomonas weihenstephanensis* (Glück et al., 2016, Volk et al., 2019).

The AprX gene is located in the *aprX-lipA2* operon, first described in *Pseudomonas fluorescens* B52 by Woods et al. 2001, comprising up to 9 different genes (Woods et al., 2001, Ma et al., 2003, Maier et al., 2020). Besides AprX, the operon genes encode for a peptidase inhibitor (AprI), a Typ I secretion system (AprDEF), two putative autotransporters with homologies to serine proteases (PrtA, PrtB), and up to two lipases (LipA1, LipA2). Moreover, the *aprX-lipA2* operon is regulated by a single promotor upstream of *aprX* (Woods et al., 2001) and can be subdivided into a conserved region (*aprXDEF*) and a region with variable gene compositions (*prtAB*, *lipA1*, *lipA2*), depending on species and partly strain (Ma et al., 2003, Maier et al., 2020). In total, 21 different operon types were identified in *Pseudomonas* so far, of which operon type I (*aprXIDEF prtAB lipA2*), found in species like *P. proteolytica*, *P.lactis,* and *Pseudomonas gessardii,* and type 2 (*aprXIDEF lipA2*), related with, e.g., *Pseudomonas fragi*, *Pseudomonas veronii,* or *Pseudomonas marginalis,* were the most frequently detected operon organizations. Whereas strains harboring the type 1 operon were generally correlated with high proteolytic potential, strains with type 2 exhibited less proteolytic activity (Maier et al., 2020). One conceivable reason for variations in proteolytic activity could be the presence of the two proteins PrtA and PrtB, which exhibit homologies to serine proteases, but whose function has not been clarified yet (Chabeaud et al., 2001, Woods et al., 2001, Ma et al., 2003).

Besides the operon structure, several external factors have been proposed to influence the production or activity of AprX in psychrotolerant pseudomonads. For instance, the temperature was described as a major influencing parameter on the AprX production (Gügi et al., 1991, Burger et al., 2000, Nicodeme et al., 2005, Baur et al., 2015a, Alves et al., 2018). Like in other psychrotolerant bacteria, e.g., *Pseudoaltermonas* and *Arthrobacter*, maximal proteolytic activity of various *Pseudomonas* species was determined mainly below their optimal growth temperature (Gügi et al., 1991, Hellio et al., 1993, Buchon et al., 2000, Nicodeme et al., 2005, von Neubeck et al., 2015, Baur et al., 2015b). In contrast to the proteolytic activity, which drops steeply above the optimal growth temperature, the activity of the lipase LipA2 decreases steadily with increasing temperatures and is assumed to be regulated at the post-transcriptional or post-translational level (Andersson, 1980, McKellar and Cholette, 1987, Woods et al.,

2001). Further, the biosynthesis of LipA2 was suspected to be modulated through differential segmental decay rates of the mRNA (Woods et al., 2001, McCarthy et al., 2004). However, the mechanisms behind it remain still unknown.

Cell density seems to be another critical factor for the AprX production, as the proteolytic activity of several *Pseudomonas* spp. was shown to be significantly increased at the late exponential or the early stationary growth phase (Matselis and Roussis, 1998, Nicodeme et al., 2005, Dufour et al., 2008, Alves et al., 2018). Moreover, studies revealed a post-transcriptional regulation of the *aprX* gene by the Gac/Rsm signal cascade, which is also activated at high cell density (Blumer et al., 1999, Heeb et al., 2002, Haas and Keel, 2003). Thereby, GacS/GacA is stimulated by a signal, which activates the transcription of the regulatory sRNAs RsmX, RsmY, and RsmZ. These sRNAs, in turn, enable the translation of the *aprX* mRNA by removing the RNA binding proteins RsmE and RsmA (Haas and Keel, 2003, Valverde et al., 2003). Nevertheless, it is unknown how exactly the Gac/Rsm cascade is induced, and the signal associated with high cell densities was not identified yet.

This study aimed to elucidate regulation mechanisms underlying AprX biosynthesis and enzyme activity in *Pseudomonas* in more detail. Therefore, the potential effects of the operon genes *aprX*, *prtA*, *prtB,* and *lipA2* were investigated in the highly proteolytic species *Pseudomonas proteolytica* by generating appropriate deletion mutants. Furthermore, the influence of temperature and growth phase on AprX production at various regulatory levels were analyzed.

Material and Methods

Plasmids, Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in table II 1. *P. proteolytica* strains were grown under aerobic conditions on tryptic soy agar (TSA, Carl Roth GmbH, Karlsruhe, Germany) or in lysogeny broth (LB, consisting of 5 g/l NaCl, 5 g/l yeast extract, 5 g/l tryptone, pH 7), which was solidified by addition of 1.5 % agar, at 30 °C for 24 h. *E. coli* strains were routinely grown on LB agar at 37 °C for 24 h. Overnight cultures of *P. proteolytica* and *E.coli* were obtained by inoculating 4 ml of tryptic soy broth (TSB, Merck Millipore KGaA, Burlington, Massachusetts) or LB with cell material received from one colony and incubated shaking (150 rpm) at 30 °C (*P.*

proteolytica) or 37 °C (*E. coli*) for 16 h. If required, LB medium was supplemented with 50 µg/ml kanamycin sulfate and/or 100 ng/µl ampicillin sodium salt.

table II 1: Plasmids and bacterial strains used in this study.

Plasmid or Strain	Description or genotype	References
***E. coli* strains**		
cc118 λpir	Δ(*ara, leu*)7697 *ara*D139Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB*(RfR) *argE(am) recA1 λpir*$^+$, cloning strain	Herrero et al., 1990
S17-1 λpir	*pro, res*$^-$ *hsdR17 (rK*$^-$ *mK*$^+$*) recA*$^-$ with an integrated *RP4-2-Tc::Mu-Km::Tn7*, Tp^r, conjugation strain	de Lorenzo et al., 1993
***P. proteolytica* strains**		
WS 5128	Wild type, raw milk isolate, Amp^R	Maier et al., 2020
WS 5616	Δ*aprX* deletion mutant of WS 5128, Amp^R	This study
WS 5617	Δ*aprX* Δ*prtAB* double deletion mutant of WS 5128, Amp^R	This study
WS 5614	Δ*prtA* deletion mutant of WS 5128, Amp^R	This study
WS 5618	Δ*prtB* deletion mutant of WS 5128, Amp^R	This study
WS 5613	Δ*prtAB* deletion mutant of WS 5128, Amp^R	This study
WS 5615	Δ*lipA2* deletion mutant of WS 5128,Amp^R	This study
Plasmids		
pNPTS 138-R6KT	*mobRP4*$^+$ ori-R6K *sacB*; Km^R	Lassak et al., 2010
pNPTS 138-R6KT_ Δ*aprX*	*mobRP4*$^+$ ori-R6K *sacB*; upstream and downstream fragments of *aprX*, Km^R	This study
pNPTS 138-R6KT_ Δ*prtA*	*mobRP4*$^+$ ori-R6K *sacB*; upstream and downstream fragments of *prtA*, Km^R	This study
pNPTS 138-R6KT_ Δ*prtB*	*mobRP4*$^+$ ori-R6K *sacB*; upstream and downstream fragments of *prtB*, Km^R	This study
pNPTS 138-R6KT_ Δ*prtAB*	*mobRP4*$^+$ ori-R6K *sacB*; upstream and downstream fragments of *prtAB*, Km^R	This study
pNPTS 138-R6KT_ Δ*lipA2*	*mobRP4*$^+$ ori-R6K *sacB*; upstream and downstream fragments of *lipA2*, Km^R	This study

Preparation of electro-competent *E. coli* cells

100 ml LB medium was inoculated with 1 ml of an overnight culture (150 rpm, 37 °C), containing the respective *E. coli* strain and cultivated (37 °C, 150 rpm) until an OD between 0.5 - 0.6. Cells were harvested in pre-chilled 50 ml falcon tubes, samples were centrifuged (7000 rpm, 4 °C, 10 min), the supernatant was removed, and the pellet was resuspended in 45 ml pre-chilled, deionized water. Further washing steps

were carried out, whereby the resuspension volume was gradually reduced, from 25 ml (pre-chilled, deionized water), over 20 ml (pre-chilled, 10 % glycerol) to 1 ml (pre-chilled, 10 % glycerol). Finally, 40 µl of bacterial suspension were filled into pre-chilled tubes, shock frozen in liquid nitrogen, and stored at – 80 °C until use.

Cloning of recombinant plasmids

All enzymes used, except for the Taq Polymerase (NEB, Ipswich, Massachusetts), were purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts) and applied according to the manufacturer's protocols. Primers were obtained from Eurofins Genomics Germany GmbH (Ebersberg, Germany).

Plasmids for the in-frame deletion mutants were constructed using the conjugative vector pNPTS138-R6KT as a backbone (table II 1). Target genes (*aprX*, *prtA*, *prtB*, and *lipA2*) and respective flanking regions were predicted as described in Maier et al. 2020, using the genome of *P. proteolytica* WS 5128 as reference (Maier et al., 2020). Upstream and downstream regions (between 449 - 932 bp) of the sequence to be deleted were amplified by standard Phusion PCR (Phusion™ High–Fidelity DNA Polymerase Kit), including chromosomal DNA of *P. proteolytica* WS 5128 as template, and respective primers, which are listed in table II 2. Standard Phusion PCR was performed in a 50 µl total reaction volume containing 10 pmol dNTPS, 25 pmol of each primer, and 0.5-1µg template. The template DNA was extracted from an overnight culture of *P. proteolytica* WS 5128 utilizing the QIAamp DNA Mini Kit (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions. Standard Phusion PCR was carried out under the following reaction conditions: Initial denaturation step at 98 °C for 30 s, 35 cycles including denaturation at 98°C for 10 s, annealing at 54 – 60 °C (depending on respective primer pair) for 15 s, and extension at 72 °C for 30 s per 1 kilobase (kb), followed by a final elongation step at 72 °C for 10 min.

For the deletion of the two genes *prtAB,* the upstream flanking region of *prtA* and the downstream region of *prtB* were amplified, using the primers C13/C14 and C15/C16, respectively (table II 2). Amplified fragments were separated via gel-electrophoresis (1 % agarose gel), extracted with the QIAquick Gel Extraction Kit (Qiagen N.V., Hilden, Germany) and digested with XBaI. The relating upstream and downstream fragments were ligated using T4 DNA ligase (total volume of 20 µl, including 2 µl Taq buffer, 8.5 µl of each fragment and 1µl T4 ligase) at 16 °C overnight. Only the flanking sites of

prtA, which were elongated by complementary overhangs, were fused scar-less, applying an overlap Phusion polymerase PCR with external primers (C5 and C8) and amplified flanking regions as templates. Constructs for deletion of *aprX*, *prtB*, *prtAB* and *lipA2* were digested with EcoRV/EcoRI and for *prtA* with BcuI/ApaI, respectively. Each fragment was then ligated into the appropriately digested and dephosphorylated (FastAP Alkaline Phosphatase) pNPTS138-R6KT backbone (total volume of 20 µl including 12 µl Insert and 5 µl digested empty backbone). Afterward, constructs were transferred into electrocompetent *E. coli* cc118 λpir cells by electroporation (0.2 cm cuvette, 1 puls, 2.5 kV) using a MicroPulser (Bio-Rad Laboratories Inc., Hercules, California), plated on LB kanamycin plates, and incubated at 37 °C overnight. Successful integration into pNPTS138-R6KT was checked by Phusion polymerase PCR, applying primers M13(-21) uni and V1. Finally, promising plasmids were reproduced in *E. coli* cultures (5 ml LB medium, cultivation at 37 °C for 16 h) and isolated using the GeneElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri). The correctness of recombinant constructs was verified by DNA sequencing at Eurofins Genomics Germany GmbH (Ebersberg, Germany) using respective primers listed in table II 2.

table II 2: Primer used for cloning, sequencing, qPCR, and RNA quality control are listed. Restriction sites are indicated in grey, and overlaps (OL) for scarless cloning are underlined. Respective restriction enzymes and amplicon characteristics are noted.

Primer	Sequence (5' → 3')	Restriction/ Overlap	Amplicon description
Primers for cloning			
C1	GCA GAA TTC AGG ATT TGC CCC TCA GTA CG	EcoRV	627 bp fragment upstreamof the *aprX* gene
C2	GCA TCT AGA AAA CGT ACT TCC TTG TTT GCA AGT G	XBaI	
C3	GCA TCT AGA TCA AGG CGT AGG ACC TGT GGT	XBaI	505 bp fragment downstream of the *aprX* gene
C4	GCA GAT ATC GGC ATT CAT CAG CCA GAT ACC ATC	EcoRI	
C5	GGA ACT AGT ACA CCT ACA ACC AGC GCT ACG	BcuI	512 bp fragment upstreamof the *prtA* gene
C6	AGA GCC GGC GTA CCA GCT CCT GCC GCA AGT GGT GAC CTT CCT CAT TTT TAT TGT GTG G	OL	
C7	ACC CAC ACA ATA AAA ATG AGG AAG GTC ACC ACT TGC GGC AGG AGC TGG TA	OL	500 bp fragment downstream of the *prtA* gene
C8	GCA GGG CCC ACG GTG TAC CGT CAT AGC GG	ApaI	
C9	CCA TGT GCC TGT GAC GG	*	932 bp fragment upstreamof the *prtB* gene
C10	GCA TCT AGA GGT GAC CTT CCT TAG TTT TGT TGC	XBaI	
C11	GCA TCT AGA GTT TTA CCC TGT AGG CGC	XBaI	

C12	GCA GAA TTC AGG TGT GGT TGC TCA AGT CC	EcoRI	772 bp fragment downstream of the *prtB* gene
C13	GCA GAA TTC ACC TAC AAC CAG CGC TAC GAC A	EcoRI	516 bp fragment upstream of the *prtA* gene
C14	GCA TCT AGA GGT GAC CTT CCT CAT TTT TAT TGT GTG	XbaI	
C15	GCA TCT AGA GTT TTA CCC TGT AGG CGC	XbaI	524 bp fragment downstream of the *prtB* gene
C16	GCA GAT ATC CGG AAG GCG ATG CCG ATT TC	EcoRV	
C17	GCA ACT AGT AAG ACC TTC AAC CTC ACC GAC C	EcoRI	520 bp fragment upstream of the *lipA2* gene
C18	GCA TCT AGA AGC CGT TTT GCT GAT ACC CC	XbaI	
C19	GCA TCT AGA GCG CAA TAC CTT CCT GTT C	XbaI	450 bp fragment downstream of the *lipA2* gene
C20	GCC GAT ATC TAC TCA GTA CCT GAC CAC CG	EcoRV	
Primers for mutant/construct verification			
M13(-21) uni	TGT AAA ACG ACG GCC AGT		amplicon to verify pNPTS 138-R6KT_ ΔTG
V1	CACT TTA TGC TTC CGG CTC		
V2	AAA TAA CCA ACT TCC CGC C		amplicon to verify *P. proteolytica* Δ*aprX*
V3	CTC CAC CAC CAA AGC AAA C		
V4	GGA GGG CAA GAC CCG CGA		amplicon to verify *P. proteolytica* Δ*prtA*
V5	GCT GGG CGT ATA CCG GCT GA		
V6	AAC CCT GTC TCT GAA TGA CCA		amplicon to verify *P. proteolytica* Δ*prtB*
V7	ACC TCG ACC TGG GCA CT		
V8	GGA GGG CAA GAC CCG CGA		amplicon to verify *P. proteolytica* Δ*prtAB*
V9	GCA GGA TCC GCC TCG ACT GGG CGT TCT		
V10	CCA GCA AGG AGA AAA TCC AC		amplicon to verify *P. proteolytica* Δ*lipA2*
V11	CAC CGA ATT ATC GCC GAA C		
Primers for qPCR			
16_qPCR_F	CCT GGG AAC TGC ATY CAA AAC T		215 bp fragment within the 16S rDNA gene
16_qPCR_R	TCC CAA CGG CTA GTT GAC ATC		
aprX_qPCR_F	ACT GGG GCG AAA ACA ACA C		119 bp fragment within the *aprX* gene
aprX_qPCR_R	GTT GTA GTT GGC ACC GTA GAG		
aprD_qPCR_F	ATC GCA GAA CGA AAC CAC		107 bp fragment within the *aprD* gene
aprD_qPCR_R	CGA TGC GGA TCA CGA TAA		
prtA_qPCR_F	AAG ATC GAC AGC ACC GTT CC		140 bp fragment within the *prtA* gene
prtA_qPCR_R	AAC ACC TGC AAG GCT TGC TC		
prtB_qPCR_F	CTG GAA CAA TAA AAA CGG CAC		100 bp fragment within the *prtB* gene
prtB_qPCR_R	TAT CTG CTC GCC ACT CAT ATA C		
lipA2_qPCR_F	GGA CTT GAG CAA CCA CAC CT		165 bp fragment within the *lipA2* gene
lipA2_qPCR_R	TTC AAT CTG TCT TCC CTG GG		
Primers for RNA quality control			
16S_27_F	AGA GTT TGA TCC TGG CTC A		674 bp fragment within the 16S rDNA gene
16S_907_R	CCG TCA ATT CMT TTG AGT TT		

*naturally with EcoRV restriction site in *prtB* flanking site, thereof no additional restriction site on primer

Construction of deletions mutants in *P. proteolytica* WS 5128 by conjugation

Recombinant plasmids were introduced into *P. proteolytica strains* (acceptor) by conjugative mating using *E. coli* S17-1 λ*pir* (donor), harboring the respective pNPTS 138-R6KT construct. Therefore, two separate cultures, one for the donor (in LB with kanamycin) and one for the acceptor (in LB medium) were prepared. For this, 6 ml medium was inoculated with 60 µl of the respective overnight culture and cultivated shaking (150 rpm, 30 °C, 5 h). Afterward, 5 ml of the acceptor culture and 1 ml of the donor culture were harvested by centrifugation (7000 rpm, RT, 3 min), and pellets were resuspended in 5 ml and 1 ml LB medium, respectively. After two further washing steps, donor and acceptor were pooled in 200 µl LB medium, spotted in the middle of an LB agar plate, and incubated at 30 °C overnight. Single-crossover integration mutants were selected by plating the cell mixture on LB agar containing ampicillin (native resistance of *P. proteolytica*) and kanamycin and incubated for 48 h at 30 °C. Single colonies were then transferred on LB agar plates containing 10 % sucrose (w/v) to select for plasmid excision and incubated at 30 °C overnight. Subsequently, the genotypes of kanamycin-sensitive cells were checked for successful deletion of the target gene by PCR using the respective primers (table II 2), and correct sequences were verified by the sequencing service of Eurofins Genomics Germany GmbH (Ebersberg, Germany). While *aprX, prtA* and *prtB* were removed entirely in the resulting deletion mutants WS 5616, WS 5614, and WS 5618, 365 bp (133 bp at 5'-end and 232 bp at 3'-end) of *lipA2* remained in the chromosome of *P. proteolytica* WS 5615. For the double deletion of *prtA* and *prtB*, both genes and their intergenic region (82 bp) were removed in the mutant strain *P. proteolytica* WS 5613. Moreover, in *P. proteolytica,* WS 5613 *aprX* was removed completely to create the triple deletion strain WS 5617, lacking *aprX*, *prtA,* and *prtB*.

Growth of *Pseudomonas proteolytica* in UHT milk and sample preparation

To determine the growth behavior of *P. proteolytica* WS5128 and the constructed deletion mutants (WS 5613-5618), an overnight culture of each strain was utilized to inoculate 50 ml UHT milk (1.5 % fat) with approximately 10^4 cells. Cultures were then

incubated shaking (150 rpm) at 12 °C or 30 °C for 72 h. Aliquots of 750 µl were harvested at different time points (0 h, 5 h, 10 h, 24 h, 35 h, 48 h, 72 h) and centrifuged (13000 rpm, 5 min) at the respective cultivation temperature (12 °C or 30 °C). Afterward, the supernatants were sterile-filtered (0.22 µm; Berrytec GmbH, Harthausen, Germany) and stored at -20 °C until use for immunodetection of AprX and quantification of the proteolytic activity by azocasein-assay. The remaining pellet was shock frozen and stored at -80 °C until RNA extraction. Simultaneously, total cell counts of the strains grown in milk were determined on TS agar at each sampling point. Doubling times (t_d) were calculated as follows:

(1) $r = \frac{\ln(\frac{N_t}{N_0})}{(t-t_0)}$ (2) $t_d = \frac{ln2}{r}$

First, growth rate (*r*) was determined from cell counts (N) at the initial (t_0) and final (t) time points. Afterward, the growth rate was used to enumerate the doubling time. For cells grown at 12 °C (t_0 = 10 h and t = 34 h) and 30 °C (t_0 = 5 h and t = 10 h) different time points were chosen to calculate t_d and three biological replicates were averaged for each strain and each condition. The normal distribution of the calculated t_d –values (three biological replicates each) for each strain at each temperature (12 °C, 30 °C) was verified with the Shapiro Wilk test. Statistical significance between the t_d –values of the wild type and different mutants were checked for both 12 °C and 30 °C applying a Welch's test. Cell counts without any further significant increase over time were defined as maximal cell counts (N_{max}), and three biological replicates were averaged for each strain and each cultivation temperature. In detail, the normal distribution (three biological replicates each) of cell counts at each sampling point was determined by a Shapiro Wilk test separately for each strain at both 12 °C and 30 °C. Then, the change in cell counts from one sampling point to the next was tested for significance using a paired t-test. If there was no significant increase of the cell counts to the next sampling point, the respective one was defined as N_{max}. This procedure was carried out for each strain at 12 °C and 30 °C.

Determination of the total and the extracellular proteolytic activity

To determine the total proteolytic activity, an agar diffusion assay was performed on skim milk agar (2x tryptic soy broth (TSB, Merck Millipore KGaA, Burlington, Massachusetts) supplemented with 10% (w/v) skim milk powder). Initially, *P.*

proteolytica strains were grown on TS-Agar. Then, one loop of cell material was suspended in 100 µl sterile deionized water and vortexed vigorously until receiving a homogenous suspension. 15 µl of the bacterial suspension were spotted on skim milk agar plates and incubated at 12 °C or 30 °C for 4 days. After 3 and 4 days the magnitude of the total proteolytic activity (Δr) was determined by subtracting the colony radius (r_{colony}) from the outer edge of the clearing zone ($r_{clearing}$). The measurement was performed in biological triplicates, and Δr values were averaged.

The extracellular proteolytic activity of *P. proteolytica* strains was determined by an azocasein assay. For this, sterile-filtered supernatant of the respective culture was diluted 10^{-1} and 10^{-2} with Ringer solution (Merck KGaA, Darmstadt, Germany). Diluted samples and freshly prepared azocasein-solution, consisting of 0.5 % (w/v) azocasein (Sigma-Aldrich, St. Louis, Missouri), 50 mM MOPS buffer (pH 6.7), and 1 mM $CaCl_2$, were pre-incubated at 40 °C for 5 min. For each sample, 100 µl were subsequently pooled with 100 µl azocasein solution and incubated shaking (600 rpm) at 40 °C for 1 h. Then, to precipitate the remaining uncleaved azocasein molecules, 20 µl TCA was added and samples were centrifuged (13000 rpm, 5 min, RT). Finally, 150 µl of each sample supernatant was mixed with 50 µl of 1 M NaOH in a microtiter plate. The absorbance at 450 nm was determined in a plate reader (Victor3, PerkinElmer Inc., Waltham, Massachusetts), and only values up to 0.59 were considered lying in the linear range of detection. The proteolytic activity (EPA) was calculated as the difference between the absorbance of the sample and a blank at 450 nm (ΔA) per hour and ml tested solution ($\mathrm{EPA} = \frac{\Delta A}{\mathrm{h\ x\ ml}}$). The measurement was performed in three technical replicates for each sample, which were averaged. For better comparability between strains and growth conditions, EPA values were normalized to the logarithm of 10^9 cells. Subsequently, the difference in EPA values between *P. proteolytica* WS 5128 and the deletion mutants (WS 5613 – WS 5618) was tested for statistical significance. Therefore, the normal distribution (three biological replicates each) of the EPA values received after 24 h, 48 h, and 72 h were determined by a Shapiro Wilk test separately for each strain at both 12 °C and 30 °C. Afterward, a one-way ANOVA with post hoc pairwise t-test including a Holm p-value adjustment was performed to compare the EPA value of wild type at each cultivation condition with the value of each of the deletion mutants.

Western blot analysis

To measure the amounts of the secreted peptidase AprX in different strains, sterile filtered supernatants of *P. proteolytica* cultures were obtained as described for the azocasein assay, and immunoblotting was performed as described previously (Volk et al., 2021). In brief, for each sample, 4 µl supernatant were mixed with 4 µl deionized water and 2 µl sample buffer (0.0625 M Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 0.001 % bromophenol blue). Afterward, the mixtures were loaded and separated on an SDS-PAGE gel (12.5 %, 120 V, 2 h) as described by Laemmli (Laemmli, 1970). Protein size was checked by applying 3 µl of the Color Prestained Protein Standard (NEB, Frankfurt am Main, Germany). Additionally, 5µg purified AprX protein isolated from *P. lactis* DSM 29167 was loaded as a positive control. After electrophoretic separation, semi-dry blotting was performed using a nitrocellulose membrane (Amersham™ Protran® 0.2 *µ*m NC, GE, Munich, Germany) and filter papers (Extra Thick Blot Paper, Bio-Rad, Munich, Germany), which were pre-saturated with transfer buffer (25 mM Tris, 192 mM Glycine, pH 8). Afterward, semi-dry blotting (40 min, 11 V) was conducted using the Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad, Munich, Germany). The membrane was then blocked with blocking solution (skim milk powder 5 % (w/v) diluted in PBS, Tween-20 (0.05 % (v/v)) overnight at 5 °C. and afterward treated three times for 10 min with washing solution (PBS containing Tween 20). Then the primary polyclonal antibody (anti-*P.lactis*-AprX; 0.2 µg*mL^{-1} in PBS, (Volk et al., 2021) was added, and the membrane was incubated shaking (50 rpm) on the TR-250 shaker (Infors AG, Bottmingen, Switzerland) for 2.5 h at 20 °C. Three further washing steps were performed in PBS containing Tween 20 for 10 min, after which the membrane was incubated in PBS containing the HRP-conjugated secondary antibody (anti-rabbit IgG from donkey; GE, Munich, Germany; diluted 1:5000 in PBS) at 20 °C for 1.5 h. AprX bands were visualized on the membrane by applying the ClarityTM Western ECL Substrate Kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. The incubation time was adapted to the respective protein concentration using the imagining system ChemiDoc Touch (Bio-Rad, Munich, Germany) to visualize the protein bands. Consequently, samples cultivated at 12 °C were incubated for 30 s, ones at 30 °C for 300 s.

RNA extraction and reverse transcription

Cell pellets, received from 750 µl culture, were pre-treated and enzymatically lysed as follows: Each pellet was resuspended in 1 ml RNAprotect (Qiagen N.V., Hilden, Germany), incubated on ice for 5 min and 350 µl of the suspension transferred to a new tube for further processing. After that, the sample was centrifuged (13000 rpm, 4 °C, 10 min) and the supernatant was removed. The remaining pellet was washed with 1 ml ringer solution and 100 µl EDTA (0.5 M) followed by a further centrifugation step (13000 rpm, 4 °C, 10 min). Subsequently, the supernatant was discarded, cells were enzymatically lysed by adding 200 µl Lysozyme solution (15 mg Lysozyme (Carl Roth GmbH, Karlsruhe, Germany) solved in TE buffer) and 15 µl proteinase K (Qiagen N.V., Hilden, Germany) and incubating for 15 min at RT under occasional vortexing. RNA was then extracted with the miRNeasy Mini Kit (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions and finally eluted with 50 µl RNA-free water. In the following, 30 µl extracted RNA was treated with Turbo-DNA-*free*™ Kit (Invitrogen AG, Carlsbad, California) as recommended by the producer for 30 min to remove remaining DNA. To ensure total DNA digestion, a control PCR, following Taq Polymerase (NEB, Ipswich, Massachusetts) standard procedure was conducted utilizing 16S_27_F and 16S_907_R (table II 2) as primers. Control PCR was performed in a total volume of 25 µl containing 5 pmol dNTPs, 12.5 pmol for each primer, and 0.1 µl of the digested RNA sample as template. Control PCR was carried out under the following reaction conditions: Initial denaturation step at 95 °C for 30 s, 30 cycles including denaturation at 95°C for 30 s, annealing at 48 °C for 30 s and extension at 68 °C for 1 min followed by a final elongation step at 68 °C for 5 min. After successful digestion, RNA concentration was determined using a spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, Delaware). For cDNA synthesis, 1 µg RNA was converted to cDNA in a 20 µl mixture using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, California), according to the manufacturer's instructions.

Quantitative PCR

To quantify the mRNA level of selected target genes (*aprX*, *aprD*, *prtA*, *prtB*, *lipA2*) a quantitative PCR (qPCR) was performed. Therefore, the genome sequence of *P. proteolytica* WS 5128 was used for primer design, and appropriate primer binding regions were manually identified (table II 2). Each primer pair was checked for self-

and cross dimer formation with Multiple Primer Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts). Hairpin formation and annealing temperatures were determined using OligoCalc (Kibbe, 2007). Annealing temperatures of the designed oligonucleotides were between 48 °C and 55 °C, GC-content reached from 43 % to 67 %, and the length varied between 18 and 25 nucleotides. The optimal annealing temperature was determined for each primer pair employing qPCR with different temperatures (58 °C, 60 °C, 62 °C), including a subsequent melting curve. As template, cDNA received for *P. proteolytica* WS 5128, cultivated at 12 °C for 48 h, was applied. For the determination of the amplification efficiency, qPCR was performed with each primer pair using a 5-fold dilution series (from 100 ng/µl to 0.16 ng/µl) of *P. proteolytica* WS 5128 cDNA as template. The following formula was used to calculate the amplification efficiency (*E*):

$$E = 10^{\frac{-1}{slope}} - 1.$$

All qPCR reactions were performed with the SsoAdvance ™ Universal SYBR®Green Supermix (Bio-Rad Laboratories Inc., Hercules, California) in a 10 µl total reaction volume and measurements were performed with a CFX96/C1000 Touch™ (Bio-Rad Laboratories Inc., Hercules, California). Data were visualized using the CFX Maestro™ software (Bio-Rad Laboratories Inc., Hercules, California). Each 10 µl reaction mixture contained 5 µl SYBR reaction buffer, 1 pmol of each primer (working concentration) and 2 µl cDNA as template. The applied program was as follows: Initial denaturation step at 95 °C for 30 s and 35 cycles, including denaturation at 95 °C for 15 s and annealing/extension for 30 s at 60 °C. All measurements were carried out in 3 technical replicates.

Relative transcription analysis

Relative expression of the target genes (*aprX*, *aprD*, *prtA*, *prtB*, *lipA2*) in different strains (*P. proteolytica* WT, *P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, and *P. proteolytica* Δ*lipA2*) and at each growth condition was determined using the Relative Expression Software Tool-Multiple Condition Solver (REST-MCS) version 2. The software is based on an efficiency corrected calculation model published by Pfaffl (2001, 2002):

$$ratio = \frac{(E_{target})^{\Delta C_{q,target}\ (MEAN\ control - MEAN\ sample)}}{(E_{reference})^{\Delta C_{q,reference}\ (MEAN\ control - MEAN\ sample)}}$$

First, the difference between the quantification cycle (C_q) values of the chosen control and the sample was determined for the reference gene (16S rRNA, $\Delta C_{q,\ reference}$) and the target gene (*aprX*, *aprD*, *prtA*, *prtB* or *lipA2*, $\Delta C_{q,\ target}$) to normalize alterations in cDNA amounts. To exclude differences based on varying primer binding, the amplification efficiencies (E) of the applied primer pairs were included in the calculation of the relative expression. The choice of control was thereby dependent on the experiment. To compare the transcription behavior of *P. proteolytica* WS 5128 wild type at different growth conditions, *P. proteolytica* WS 5128 grown at 30 °C for 24 h was used as a control. When analyzing the relative expression between *P. proteolytica* wild type and the constructed deletion mutant at a specific temperature, *P. proteolytica* WS 5128 grown at the respective temperature for 24 h was defined as control. Statistical tests were performed within REST-MCS implemented two-sided pairwise fixed reallocation randomization test. Here, a distribution-independent statistical test was conducted that reallocates the paired expression ratios of the reference gene and the respective target gene randomly to the two compared groups (e.g., comparison of *P. proteolytica* WS 5128 cultivated at 30°C for 24 h (group 1) and 35 h (group 2) (Pfaffl et al., 2001, 2002). This reallocation was performed 2000 times for each compared group of two.

Statistical tests

Shapiro Wilk test was applied to test for normal distribution of the data. To determine differences of the doubling (t_d) time at 12 °C and 30 °C between *P. proteolytica* WS 5128 and mutant strains, a Welch's test was performed. For the verification of the maximum cell counts of each analyzed strain, a paired t-test was applied to compare the increase of cell counts from one sampling point to another. To check for significant differences in EPA values between *P. proteolytica* WS 5128 and mutant strains at different sampling points at 12 °C and 30 °C, a one-way ANOVA with post hoc pairwise t-test including a Holm p-value adjustment was performed. All tests described so far were carried out with Rstudio version 4.0.3 (R Core Team, 2019). Moreover, statistics for relative transcription analysis were performed with a distribution-independent two-sided pairwise fixed reallocation randomization test implemented in the relative

expression software tool (REST-MCS). Therefore, 2000 reallocations were performed (Pfaffl et al., 2002). P-values indicated the degree of significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.01$).

3. Results

Construction of deletion mutants in *P. proteolytica* WS5128 and growth in UHT milk

All deletion mutants originated from *P. proteolytica* WS 5128, a wild type strain with operon type 1 (*aprXIDEF prtAB lipA2*) and high proteolytic activity in milk. To study the influence of the operon genes *prtA, prtB,* and *lipA2,* located in the variable region of the *aprX-lipA2* operon, several deletion mutants of *P. proteolytica* WS 5128 were constructed. In total, three in-frame single mutant strains (*P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*lipA2*), one double deletion mutant (*P. proteolytica* Δ*prtAB*), and one triple deletion mutant (*P. proteolytica* Δ*aprX* Δ*prtAB*) were successfully generated by conjugative mating. Additionally, *P. proteolytica* Δ*aprX* was created to verify AprX being the sole secreted, caseinolytic peptidase in *P. proteolytica*.

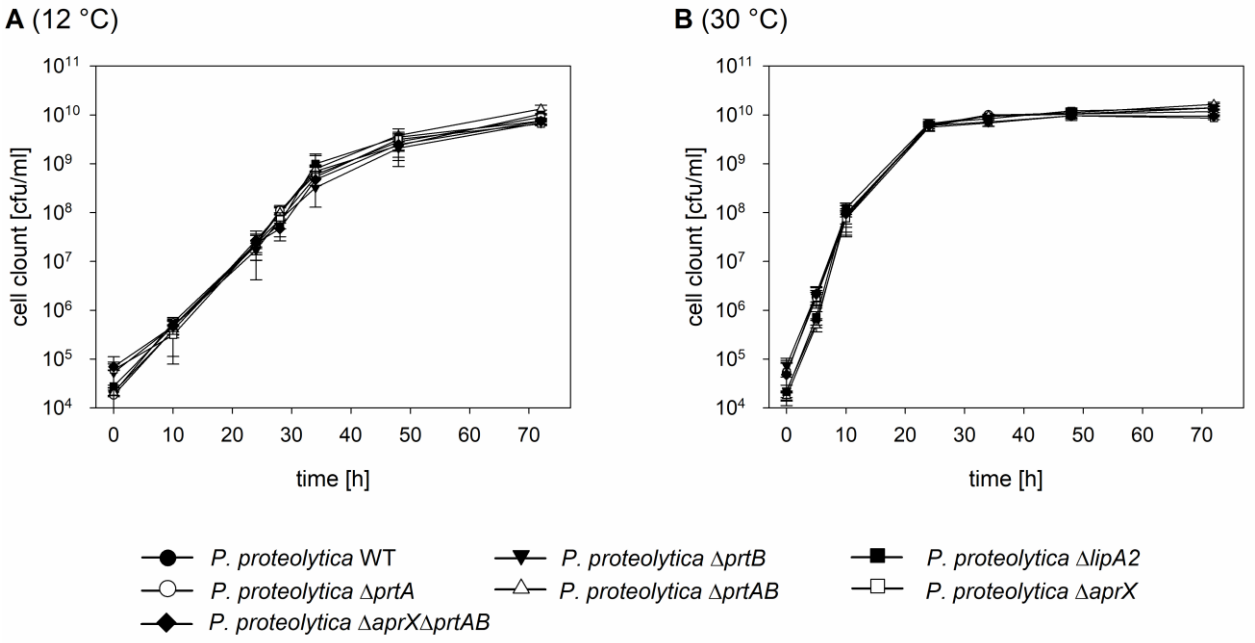

figure II 1: Growth behavior of *P. proteolytica* wild type (WT) and six deletion mutants (*P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*, *P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*aprX*Δ*prtAB*), grown in UHT milk at 12 °C (A) and 30 °C (B) for 72 h. Mean values were calculated from three biological replicates and standard deviation is indicated by error bars.

To exclude variation in the AprX biosynthesis based on various growth behavior, the *P. proteolytica* deletion mutants and the wild type strain (WT) were cultivated in milk (1.5 % UHT milk) at 12 °C and 30 °C for 72 h (figure II 1). The wild type reached the stationary phase after 24 h at 30 °C and after 48 h at 12 °C. At both temperatures, all six deletion mutants exhibited wild type-like growth.

Moreover, at both temperatures, doubling times (t_d) and maximal cell counts (N_{max}) of the wild type and the mutant strains did not differ significantly (table II 3). Cells grown at 30 °C exhibited a shorter ($t_{d,\ 30°C}$ = 39.4 – 55.7 min) doubling time compared to ones cultivated at 12 °C ($t_{d,\ 12°C}$ = 130.4 – 152.7 min). Consequently, the early stationary growth phase was reached after approx. 24 h at 30 °C and between 35 - 48 h at 12 °C, respectively. Due to the optimal growth temperature of *Pseudomonas* spp. at 25-30 °C, these results were not surprising (Gügi et al., 1991). At 30 °C, maximal cell counts were determined for all strains after 24 h ($5.6*10^9$ - $6.7*10^9$ cfu/ml), except for *P. proteolytica* Δ*prtA* ($1.0*10^{10}$) and *P. proteolytica* Δ*prtAB* ($9.7*10^9$), which reached maximal cell counts after 35 h. At 12 °C, maximal cell counts were generally detected after 72 h ($6.6*10^9$ - $1.3*10^{10}$ cfu/ml) for all strains.

table II 3: Doubling times (t_d) and maximal cell counts (N_{max}) of *P. proteolytica* WS 5128 (WT) and six deletion mutants (*P. proteolytica* Δ*prtA, P. proteolytica* Δ*prtB, P. proteolytica* Δ*prtAB, P. proteolytica* Δ*lipA2, P. proteolytica* Δ*aprX, P. proteolytica* Δ*aprX*Δ*prtAB*) at 12 °C and 30 °C. Doubling time was calculated between 10 h and 34 h (12 °C) or 5 h and 10 h (30 °C) cultivation time. Maximal cell counts were detected after 72 h (12 °C) or 24 h (30°C). For *P. proteolytica ΔprtA* and *P. proteolytica ΔprtAB* maximal cell counts were determined after 34 h at 30°C (‡). All values were averaged from three biological replicates.

	WT	Δ*aprX*	Δ*prtA*	Δ*prtB*	Δ*prtAB*	Δ*lipA2*	Δ*aprX*Δ*prtAB*
$t_{d,\ 12\ °C}$ [min]	133.1	133.9	152.7	144.4	136.2	137.7	130.4
$t_{d,\ 30\ °C}$ [min]	41.7	55.7	55.1	54.9	53.6	39.4	40.4
$N_{max,\ 12°C}$ [cfu/ml]	$1.1*10^{10}$	$9.0*10^9$	$6.9*10^9$	$1.3*10^{10}$	$7.5*10^9$	$6.6*10^9$	$7.4*10^9$
$N_{max,\ 30°C}$ [cfu/ml]	$6.6*10^9$	$5.6*10^9$	$1.0*10^{10}$ ‡	$6.3*10^9$	$9.7*10^9$ ‡	$6.7*10^9$	$6.1*10^9$

Effect of temperature and gene deletion on proteolytic activity and AprX amount

To analyze the effect of *aprX, prtA*, *prtB,* and *lipA2* on the proteolytic activity, *P. proteolytica* wild type and the six deletion mutant strains (were spotted on skim milk agar and cultivated at 12 °C and 30 °C for 4 days. the proteolytic activity was determined through the clearing zones' size after day 3 and day 4 (figure II 2).

At 12 °C, the total proteolytic activity of *P. proteolytica* wild type increased 33 % from day 3 (Δr = 6.0 mm) to day 4 (Δr = 8.0 mm). *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB,* and *P. proteolytica* Δ*lipA* behaved like the wild type, exhibiting an elevated proteolytic activity (22 - 35 %) from day 3 (Δr = 5.7 - 6.0 mm) to day 4 (Δr = 7.3 -7.7 mm). In contrast, *P. proteolytica* Δ*aprX* and *P. proteolytica* Δ*aprX*Δ*prtAB* were not caseinolytic at all (figure II 2).

At 30 °C, the proteolytic activity of *P. proteolytica* wild type was higher compared to the values of the 12 °C-samples, by means clearing zones were 39 % larger at day 3 (Δr = 8.3 mm) and 17 % at day 4 (Δr = 9.3 mm). *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB,* and *P. proteolytica* Δ*lipA* did not differ from the wild type, as the size of the clearing zones was also increased by 28 - 39 % on day 3, and 22 - 35 % on day 4 compared to the respective 12 °C – values of these strains. Again, *P. proteolytica* Δ*aprX* and *P. proteolytica* Δ*aprX*Δ*prtAB* did not produce any caseinolytic activity, as observed at 12 °C (figure II 2 or data not shown).

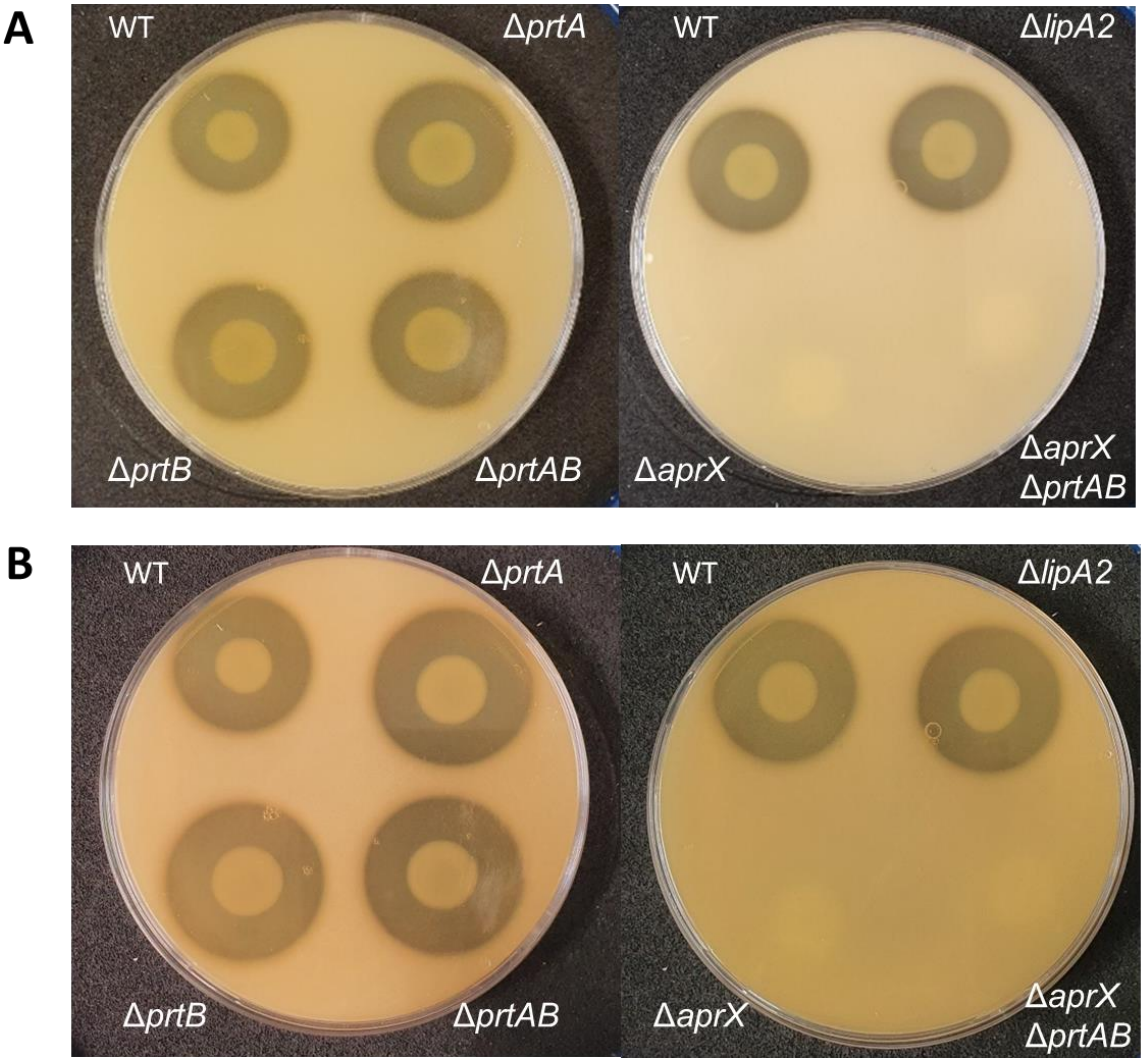

figure II 2: Total proteolytic activity of *P. proteolytica* WS 5128 and six deletion mutants (*P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*, *P. proteolytica* Δ*aprX* and *P. proteolytica* Δ*aprX* Δ*prtAB*), grown on skim milk agar at 12 °C for 3 days (A) and 4 days (B).

To examine the effect of *aprX, prtA*, *prtB,* and *lipA2* on AprX peptidase amounts and quantify the extracellular proteolytic activity (EPA), immunoblotting with a specific antibody against AprX and an azocasein assay were performed, respectively. Therefore, supernatants from cultures grown in UHT milk at two different temperatures (12 °C, 30 °C) were obtained at various time points (24 h, 48 h, and 72 h) and analyzed accordingly.

Mutant strains lacking *aprX* (*P. proteolytica* Δ*aprX* and *P. proteolytica* Δ*aprX*Δ*prtAB*) did not exhibit any proteolytic activity (figure II 3). In contrast, the other strains (*P. proteolytica* WT, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*) displayed considerable strain-dependent variations in their proteolytic behavior, also strongly affected by the cultivation temperature. In general, proteolytic activities of all strains were clearly increased at 12 °C compared to 30 °C. The most extensive variation was measured after 72 h, with enzyme activity of *P. proteolytica* wild type being 26 times larger at 12 °C than at 30 °C. In total, *P. proteolytica ΔlipA2* showed the highest proteolytic activity (1171 ΔA * h^{-1} * ml^{-1}) at 12 °C after 72 h.

At 12 °C, all strains displayed proteolytic activity initially after 48 h of cultivation, at which cells reached the early stationary phase. For the wild type strain, proteolytic activity increased 1.7-fold from 48 h (638 ΔA * h^{-1} * ml^{-1}) to 72 h (1104 ΔA * h^{-1} * ml^{-1}). The proteolytic profile of *P. proteolytica* Δ*lipA2* was comparable with that of the wild type, whereas peptidase activities of strains missing *prtA* and/or *prtB* were significantly reduced (figure II 3). After 48 h, *P. proteolytica* Δ*prtAB* exhibited 0.63-fold, and the single deletion strains (*P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB*) 0.1-fold of the wild type's activity. Interestingly after 72 h, only *P. proteolytica* Δ*prtB* (0.65-fold) and *P. proteolytica* Δ*prtAB* (0.7-fold) were less proteolytic than the wild type, while *P. proteolytica* Δ*prtA* was as proteolytic as the wild type.

At 30°C, *P. proteolytica* wild type cells were already in the stationary growth phase after 24 h and exhibited proteolytic activity (39 ΔA * h^{-1} * ml^{-1}), but no significant alteration during extended cultivation time was observed. The proteolytic profile of *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB,* and *P. proteolytica* Δ*prtAB* was wild type-like at all sampling points. The deletion mutant *P. proteolytica* Δ*lipA2*, however, exhibited

a 1.8-fold (48 h) and 2.0-fold (72 h) increase in the proteolytic activity compared to the wild type.

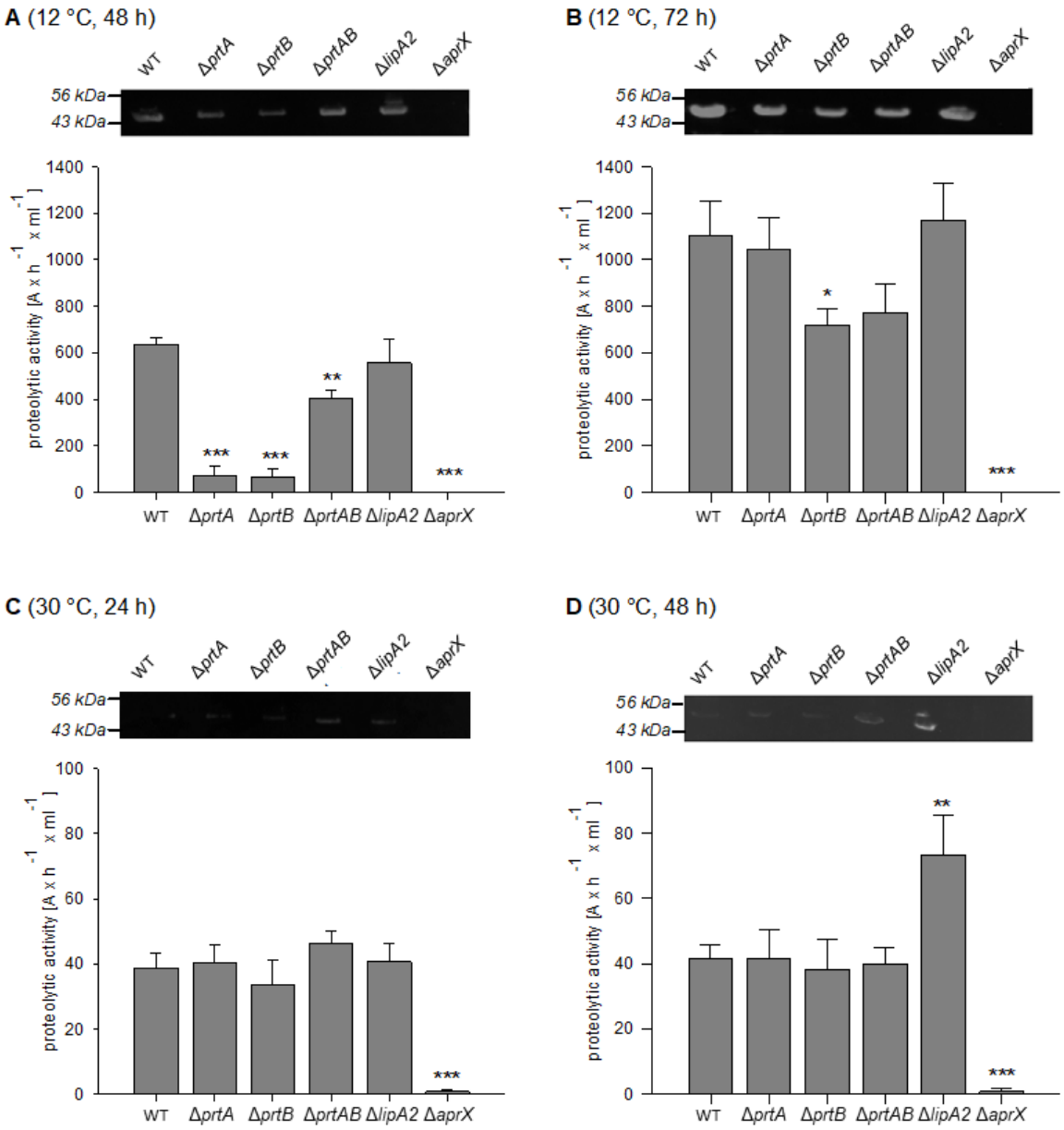

figure II 3: Proteolytic activity and corresponding AprX amounts in the supernatant of *P. proteolytica* WS 5128 (WT) and five deletion mutants (*P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*lipA2*, *P. proteolytica* Δ*aprX*), grown in UHT milk at 12 °C for 48 h (A) and 72 h (B) and at 30 °C for 24 h (C) and 48 h (D). Extracellular proteolytic activity was determined by azocasein assay. Mean values were derived from three biological replicates measured in three technical replicates. Standard deviation is indicated by error bars and for statistics a one-way ANOVA with a post hoc pair wise t-test including a Holm p-value adjustment was performed (* = p-value < 0.05; **= p-value <0.01; *** = p-value < 0.001). Respective AprX amounts of strains were determined by specific immunoblotting using a polyclonal antibody (anti-*P. lactis*-AprX). Western blot analyses were performed by Veronika Volk (Universität Hohenheim) using a single replicate.

In addition to the proteolytic activity, the secreted peptidase amounts of the strains' supernatants were determined semi-quantitatively by immunoblotting with an anti-AprX antibody. Generally, AprX amounts and enzyme activities received by the azocasein assay exhibited very similar tendencies. As expected, strains missing *aprX* did not produce any signal in the immunoblot. The other strains displayed specific bands for AprX at an appropriate size of approx. 48 kDa. In general, band intensities were elevated at 12 °C (30 s incubation time) compared to ones received at 30 °C (300 s incubation time), which were hardly visible. For 12 °C- samples, the AprX amounts were first detectable after 48 h, increasing until 72 h, whereas for 30 °C-sample weak signals already appeared at 30 °C after 24 h. Moreover, at 12 °C, *P. proteolytica* Δ*prtA* (at 48 h), *P. proteolytica* Δ*prtB* (at 48 and 72 h), and *P. proteolytica* Δ*prtAB* (at 48 and 72 h) showed decreased AprX amounts compared to *P. proteolytica* wild type, which is in good agreement with values received by azocasein assay. Besides, at 30 °C, only *P. proteolytica* Δ*lipA2* secreted more AprX than the *P. proteolytica* wild type after 48 h, which was also consistent with the proteolytic activity determined by azocasein assay (figure II 3).

Transcription analysis of *aprX-lipA2* operon genes in *P. proteolytica* WS 5128 and its deletion mutant strains

The transcript levels of selected operon genes (*aprX*, *aprD*, *prtA*, *prtB*, *lipA2*) were determined in *P. proteolytica* WS 5128 to identify possible regulatory effects of cultivation temperature and growth phase on the transcription of the *aprX-lipA2* operon. For this purpose, RNA was isolated from cells cultivated in UHT milk for different time periods at 12 °C (24 h, 48 h, and 72 h) and 30 °C (24 h, 35 h, and 48 h), followed by an RT-qPCR to quantify different target genes (figure II 4).

Generally, transcript amounts of *aprX*, *aprD*, *prtA,* and *prtB* were 10 to 500-fold higher for cells grown at 12 °C compared to 30 °C- samples. Interestingly, the differences in the transcription levels between 12 °C and 30 °C gradually decreased from the proximally located *aprX* gene to the distal *lipA2* gene. Only *lipA2* did not exhibit significant variations in the transcription level comparing 12°C- and 30 °C-samples.

At 12 °C, the transcript level of *aprX* increased in average 17-fold from 24 h (exponential growth phase) to 72 h (early stationary growth phase), whereas transcription of the downstream located genes *aprD*, *prtA*, *prtB* and *lipA2* exhibited only slight variations from 24 h to 72 h cultivation time.

At 30 °C, the amount of *aprX*, *aprD*, *prtA* and *prtB* mRNA increased from 24 h (early stationary growth phase) to 35 h (stationary growth phase) only between 2.5-fold and 4.8-fold. Interestingly, at 48 h, the mRNA of *aprX* decreased 0.9-fold compared to the initial transcription at 24 h. In contrast, the mRNA amount of *aprD*, *prtA* and *prtB* remained largely stable between 35 h to 48 h. However, the transcript level of *lipA2* remained stable over 35 h before it declined by approx. 0.9-fold after 48 h, similar to *aprX* transcription (figure II 4).

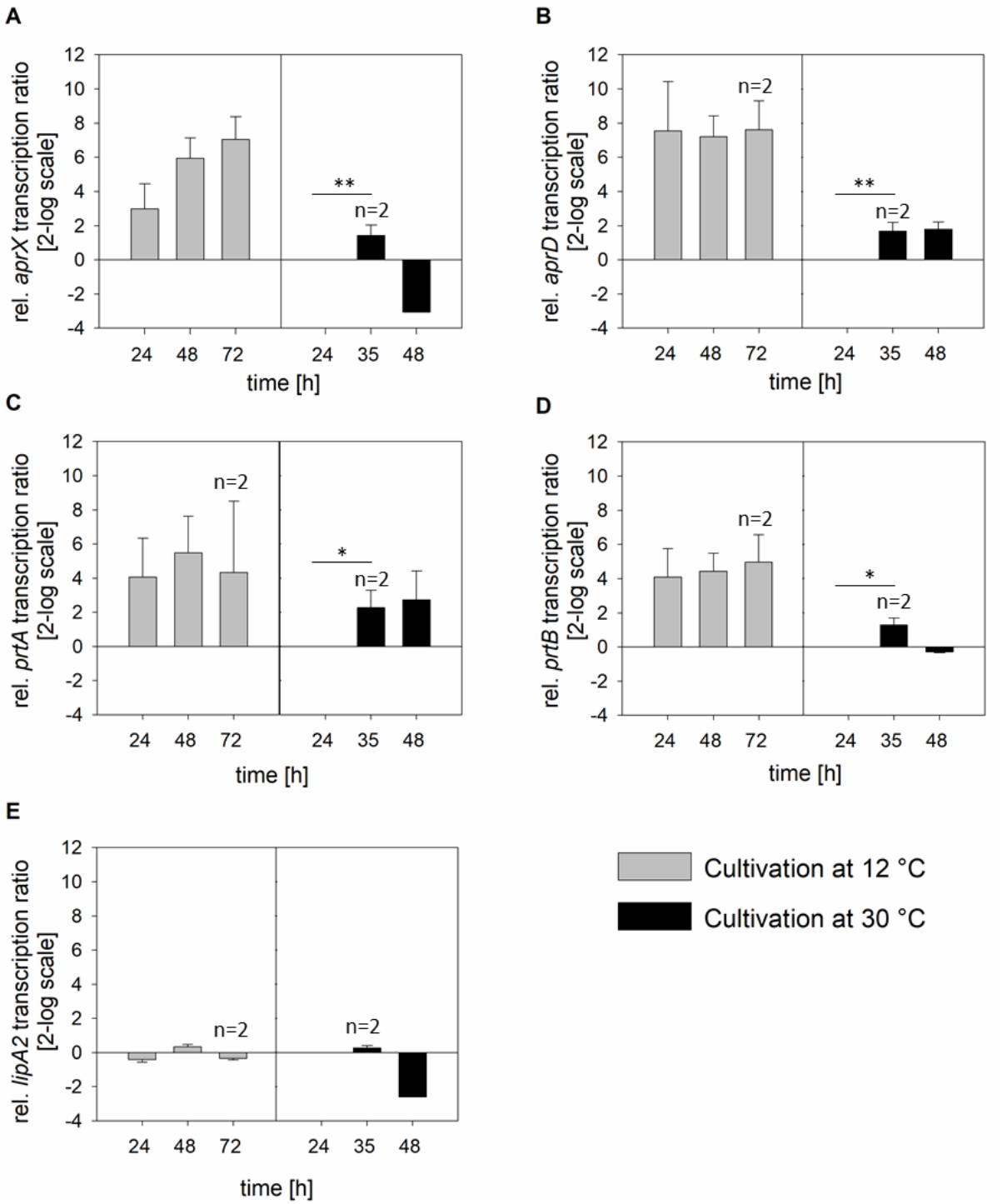

figure II 4: Relative transcription levels of *aprX* (A), *aprD* (B), *prtA* (C), *prtB* (D), and *lipA2* (E) of *P. proteolytica* WS 5128 cultivated in UH-milk at 12 °C (grey bars) and 30 °C (black bars) up to 72 h. Cells were harvested at three different time points (12 °C: after 24 h, 48 h and 72 h; 30 °C: after 24 h, 35 h, and 48 h). RNA was isolated from cell pellets, and mRNA levels of target genes were determined by RT-qPCR. The expression ratios were calculated in relation to the control sample, for which *P. proteolytica* WS 5128 cultivated at 30°C for 24 h was chosen and calculated by REST-MCS (Pfaffl et al., 2002). Values were normalized using transcript levels of 16S rRNA as reference gene. Mean values were derived from three biological replicates measured in three technical replicates, if not indicated otherwise (n = 2). Standard deviation is shown as error bars and statistics were performed by pairwise fixed reallocation randomisation test (* = p-value < 0.05; **= p-value <0.01) with 2000 randomisations (Pfaffl et al., 2002).

Next, *aprX*-, *aprD*-, *prtA*-, *prtB*- and *lipA2* transcription of the five deletion mutant strains (*P. proteolytica* Δ*aprX*, *P. proteolytica* Δ*prtAB*, *P. proteolytica* Δ*prtA*, *P. proteolytica* Δ*prtB,* and *P. proteolytica* Δ*lipA2*) was compared to the transcript levels of *P. proteolytica* wild type, in order to clarify the contribution of the deleted gene product on the expression of the *aprX-lipA2* operon. Again, strains were grown in UHT milk at 12 °C and 30 °C, harvested at specific time points, and the transcription of selected genes were determined by RT-qPCR. All deletion mutant strains behaved like the wild type, namely reached the early stationary growth phase after 24 h at 30 °C and between 35 h and 48 h at 12 °C.

At 12 °C, *P. proteolytica* Δ*aprX* showed significantly reduced transcript amounts for all analyzed target genes, except for *lipA2,* after 24 h, in comparison to the gene transcription of the wild type (figure II 5). During further cultivation, *P. proteolytica* Δ*aprX* behaved similarly to the wild type regarding operon gene transcription, except for the mRNA level of *aprD,* which remained decreased, exhibiting only 17 % of the wilt type mRNA level at 72 h of cultivation. The double deletion mutant *P. proteolytica* Δ*prtAB* showed similar transcription trends like *P. proteolytica* wild type for all target genes, excluding the mRNA level of *aprX*, which decreased in this mutant around 0.9-fold at 72 h. Interestingly, the respective single deletion mutants (*P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB*) exhibited clearly lower transcript amounts for all target genes than the wild type strain after 24 h: For *P. proteolytica* Δ*prtA,* the mRNA levels of *aprX*, *aprD*, and *prtB* were significantly reduced between 29- and 33-fold and 8-fold for *lipA2*. For *P. proteolytica* Δ*prtB*, minimized transcripts for *aprX* (146-fold), *aprD* (192-fold)*, prtA* (33-fold) and *lipA2* (13-fold) were observed. However, both single deletion mutants reached wild type-like values for all target genes after 48 h and 72 h of cultivation. Only for *lipA2*, the mean transcript amounts exceeded wild type transcript levels by 8.7-fold (*P. proteolytica* Δ*prtA*) and 15.8-fold (*P. proteolytica* Δ*prtB*), respectively, after 72h of cultivation. The mutant strain *P. proteolytica* Δ*lipA2* displayed for all target genes wild type-like mRNA levels at all sampling points (figure II 5).

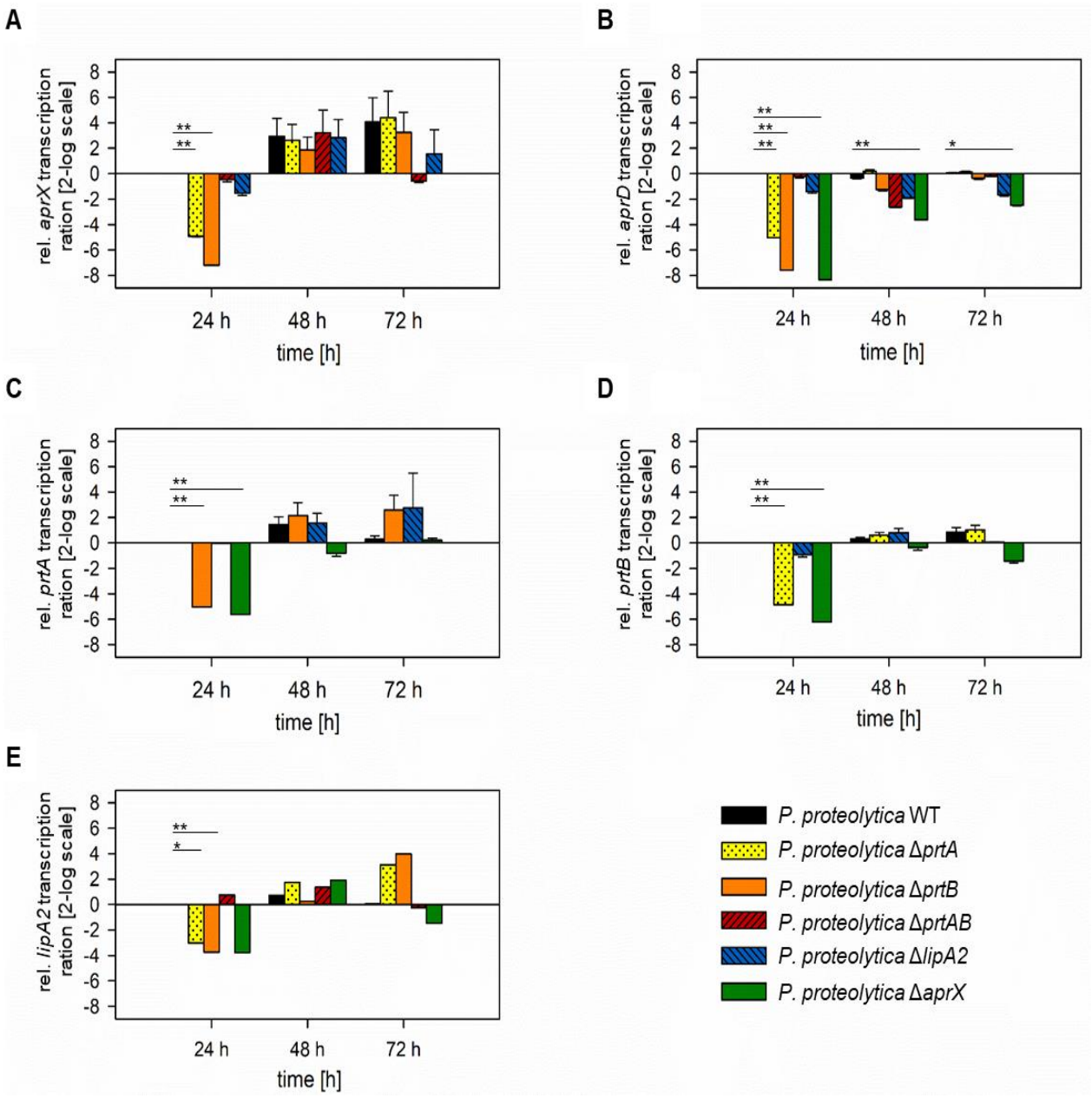

figure II 5: Relative transcription levels of *aprX* (A), *aprD* (B), *prtA* (C), *prtB* (D) and *lipA2* (E) in *P. proteolytica* WS 5128 and five deletion mutants (*P. proteolytica ΔaprX, P. proteolytica ΔprtAB, P. proteolytica ΔprtA, P. proteolytica ΔprtB,* and *P. proteolytica ΔlipA2*) cultivated in UHT milk at 12 °C for 72 h. Cells were harvested after 24 h, 48 h and 72 h, RNA was isolated from cell pellets and RT-qPCR used to determine mRNA levels of target genes. The expression ratios were calculated in relation to the control sample (= transcripts of WT grown at 12 °C for 24 h) and calculated by REST-MCS (Pfaffl et al., 2002). Values were normalized using transcript levels of 16S rRNA as reference gene. Mean values were derived from three biological replicates measured in three technical replicates, except for all strains after 72 h (n=2), *P. proteolytica ΔprtA* after 48 h (n=1), and *P. proteolytica ΔprtB* after 48 h (n=2). Standard deviation is shown as error bars and statistics were performed by pair wise fixed reallocation randomisation test (* = p-value < 0.05; **= p-value <0.01) with 2000 randomisations (Pfaffl et al., 2002).

At 30° C, transcript amounts of the mutant strains were generally reduced compared to mRNA amounts from 12 °C-samples, as it was also seen for the wild type strain *P. proteolytica* WS5128 (figures II 6 and II 4). Moreover, after 48 h of cultivation, transcripts of *aprX* and *lipA2* dropped below the initial value (after 24 h) in all strains.
Gene transcription of *P. proteolytica* Δ*aprX* was comparable to transcripts of the wild type, except for significantly reduced *aprD* (4.3-fold) and *prtA* (7.6-fold) transcript levels after 48 h. The double deletion mutant strain *P. proteolytica* Δ*prtAB* showed similar transcription trends like *P. proteolytica* wild type for all target genes at all sampling points. However, the single deletion mutants (*P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB*) behaved partly different: *P. proteolytica* Δ*prtA* showed elevated mRNA amounts for *aprX* (6.7-fold) and *aprD* (11.5-fold) after 24 h, but reduced transcript levels of *prtB* (0.48-fold) and *lipA2* (0.92-fold) at 35 h (figure II 6). For *P. proteolytica* Δ*prtB* increased transcript amounts of *aprX* (3.9-fold), *aprD* (5.5-fold) and *prtA* (5-fold) transcription levels after 24 h were observed.
Moreover, *P. proteolytica* Δ*lipA2* displayed increased mRNA levels of *aprD* (3.2-fold) and *prtA* (3.4-fold) after 24 h, *aprX* (11.7-fold) after 35 h and *prtB* after 48 h (14.6-fold) and 72 h (6.8-fold).

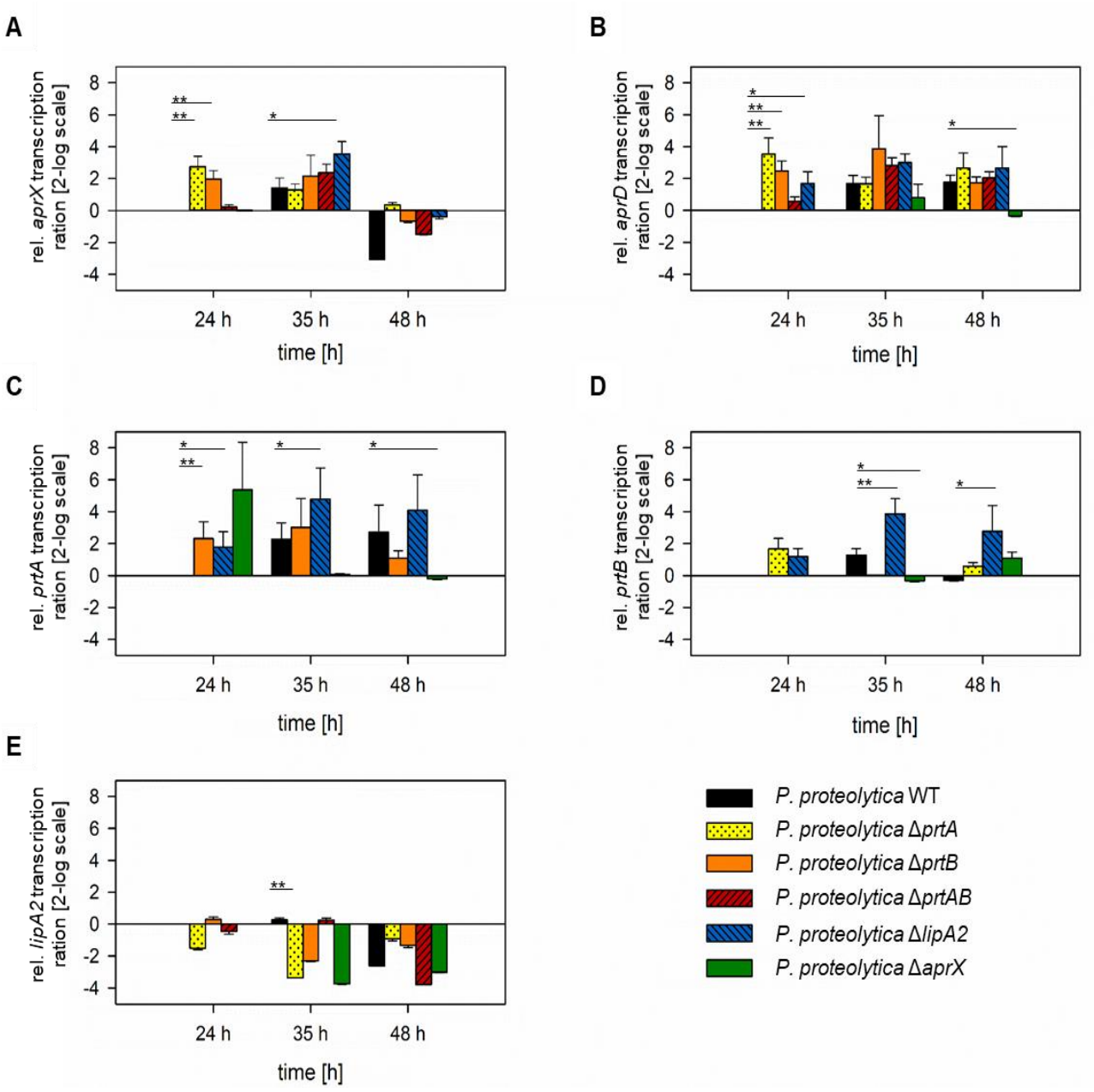

figure II 6: Relative transcription levels of *aprX* (A), *aprD* (B), *prtA* (C), *prtB* (D) and *lipA2* (E) in *P. proteolytica* WS 5128 and five deletion mutants (*P. proteolytica ΔaprX, P. proteolytica ΔprtAB, P. proteolytica ΔprtA, P. proteolytica ΔprtB,* and *P. proteolytica ΔlipA2*) cultivated in UHT milk at 30 °C for 48 h. Cells were harvested after 24 h, 35 h and 48 h, RNA was isolated from cell pellets and RT-qPCR used to determine mRNA levels of target genes. The expression ratios were calculated in relation to the control sample (= transcripts of WT grown at 30 °C for 24 h) and calculated by REST-MCS (Pfaffl et al., 2002). Values were normalized using transcript levels of 16S rRNA as reference gene. Mean values were derived from three biological replicates measured in three technical replicates, except for *P. proteolytica* WT, *P. proteolytica ΔprtAB* and *P. proteolytica ΔlipA2* after 35 h (n=2). Standard deviation is shown as error bars and statistics were performed by pair wise fixed reallocation randomisation test (* = p-value < 0.05; **= p-value <0.01) with 2000 randomisations (Pfaffl et al., 2002).

In summary, transcription analysis revealed generally elevated mRNA levels of *aprX*, *aprD*, *prtA* and *prtB* for the 12 °C- samples compared to 30 °C-samples. Interestingly, the transcription levels of *lipA2* did not differ at both temperatures. Further, the transcription of the *aprX-lipA2* operon genes already occurred at the exponential growth phase, and mRNA levels did not alter significantly during the stationary phase at 12 °C. Moreover, the transcript amounts decreased from the proximally located *aprX* gene to the distal *lipA2* at both temperatures. Whereas *P. proteolytica* Δ*prtAB*. showed wild type-like transcription patterns of *aprX*, *aprD*, *prtA*, *prtB* and *lipA2*, the single deletion mutants *P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB* exhibited increased (30 °C) or decreased (12 °C) transcript levels of the operon genes after 24 h compared to the wild type transcripts. For *P. proteolytica* Δ*lipA2,* elevated *aprX*, *prtA*, and *prtB* transcripts were only observed at 30 °C cultivation, while at 12 °C mRNA levels were wild type-like.

4. Discussion

The species-specific gene composition of the *aprX-lipA2* operon in *Pseudomonas* was shown to correlate partly with the extent of proteolytic activity (Maier et al., 2020), indicating a potential role of the operon's genes in the production or regulation of AprX expression. Therefore, this study aimed to determine the possible influence of selected operon genes (*aprX*, *prtA*, *prtB,* and *lipA2*) on the transcription of the *aprX-lipA2* operon, the amount of produced AprX, and the caseinolytic activity of the proteolytic strain *P. proteolytica* WS 5128. Moreover, the influence of different growth temperatures (12 °C and 30 °C) and cell densities on the biosynthesis of AprX were investigated.

The effect of temperature on the proteolytic activity of *P. proteolytica* WS 5128 was first analyzed by an agar diffusion assay, exhibiting slightly reduced clearing zones for the 12 °C-samples compared to 30 °C-samples. These results correspond with findings of Gügi et al., showing increased clearing zones at 30 °C compared to 8 °C for *P. fluorescence* MF0, and of Quintieri et al., observing elevated caseinolytic activity for three different *P. lactis* strains at 30 °C when compared to 15 °C (Gügi et al., 1991, Quintieri et al., 2020). However, since the optimal substrate turnover rate of the peptidase AprX lies between 35 °C and 40 °C, the casein hydrolysis through AprX is

decelerated at 12 °C (Dufour et al., 2008, Stoeckel et al., 2016, Volk et al., 2019), which is why only limited conclusions can be drawn from these experiments about the amount of secreted enzymes at different growth temperatures.

Thus, the extracellular proteolytic activity (EPA) and the AprX amounts were measured by azocasein assay and immunoblotting, respectively. For *P. proteolytica* WS 5128, 30 °C-samples showed clearly weaker EPA as well as lower peptidase amounts than the 12 °C-samples in stationary growth phase. These results agree with previous studies showing that maximum EPA was below the optimal growth temperature of psychrotolerant pseudomonads. For example, Nicodeme et al. determined elevated EPA for *P. fluorescens* CIP 6913 and *P. chlororaphis* spp. at 20 °C compared to higher temperatures (25 °C, 30 °C, 37 °C) and Hellio et al observed maximal EPA at 17.5 °C for *P. fluorescens* MF0 (Hellio et al., 1993, Nicodeme et al., 2005). In contrast, Alves et al. determined increased EPA at 25 °C compared to 10 °C for *P. fluorescens* 07A and Nicodeme at 30 °C compared to 20 °C for *P. chlororaphis* CIP 103295 (Nicodeme et al., 2005, Alves et al., 2018). These results indicate that maximum proteolytic activity is strongly temperature-dependent and strain-specific and further suggests adaptation of different strains to specific environmental conditions.

Our findings imply that AprX biosynthesis in *P. proteolytica* WS 5128 is regulated by temperature at the transcriptional level, as *aprX* mRNA amounts were generally elevated at 12 °C compared to 30 °C, despite accelerated cell growth at 30°C. Consequently, either alteration in mRNA stability or temperature-dependent transcription regulation can be assumed. Transcriptional regulation could occur through a temperature-dependent membrane-bound receptor at one end and a promotor-binding sigma factor at the other end of a signaling cascade. Such a system (PrtIR) was described in *P. fluorescens* LS107d2 as a stress response for temperatures above the growth optimum. Thereby PrtI and PrtR seem to act as transmembrane activator and ECF sigma factor, respectively (Burger et al., 2000). Besides, sequence features that enable interaction with ECF-sigma factors or δ^{70}, were predicted for the *aprX* promotor of *P. fluorescens* B52 (Burger et al., 2000, Woods et al., 2001). Accordingly, such a temperature-dependent regulatory mechanism at the transcriptional level through sigma factors is also quite conceivable in *P. proteolytica* WS 5128 and might control the *aprX* transcription positively at lower temperatures resulting in increased proteolytic activities.

Besides transcriptional regulation, studies conducted with *P. aeruginosa* spp. and *P. fluorescens* CHA0 indicated a translational regulation by temperature. The sensor kinase RetS, was attributed to interact with Gac/Rsm signaling cascade on response to temperature changes (Humair et al., 2009, Workentine et al., 2009). The Gac/Rsm is a global regulatory system induced in the early stationary phase and widely distributed among bacteria to control the formation of biofilm, the production of secondary metabolites and extracellular enzymes (Sacherer et al., 1994, Matselis and Roussis, 1998, Heeb et al., 2002, Zuber et al., 2003, Sobrero and Valverde, 2020). In *Pseudomonas,* it includes a two-component system containing a receptor (GacS) and a response regulator (GacA) (Blumer et al., 1999, Zuber et al., 2003). Phosphorylated GacA promotes the transcription of small regulatory RNAs (RmsX, RsmY, and RsmZ), which are bound by the RNA binding proteins RsmA and RsmE, resulting in translational derepression of the *aprX* mRNA (Heeb et al., 2002, Valverde et al., 2003, Kay et al., 2005, Reimmann et al., 2005, Lapouge et al., 2007). At 35 °C RetS interacts with GacS, leading to a reduced expression of sRNA (RsmX, RsmY, and RsmZ) and consequently the translation repression of *aprX* (Goodman et al., 2009, Humair et al., 2009). This effect was decreased at a lower temperature (30 °C) or if the *retS* sequence contained a point mutation (Humair et al., 2009). However, as species- or strains-specific adaptation to temperature are commonly occurring in *Pseudomonas* species, the influence of RetS or a potentially upstream sensor, adapted to low temperatures, cannot be excluded (Gügi et al., 1991, Nicodeme et al., 2005, von Neubeck et al., 2015, Alves et al., 2018). We identified a homolog of RetS in *P. proteolytica* WS 5128, showing 87 % aa similarity to the protein of *P. protegens* CH0A, but the deletion of *retS* in *P. proteolytica* WS 5128 did not increase proteolytic activities at 30 °C (data not shown). Thus, low-temperature regulation could also be independent of RetS for *P. proteolytica* WS 5128. Nevertheless, the primary effect of temperature on the AprX biosynthesis seems to be at the transcriptional level.

Besides temperature, our findings suggest an influence of the growth phase on the AprX biosynthesis of *P. proteolytica* WS 5128. The early stationary phase was reached approximately after 24 h at 30 °C and 48 h at 12 °C. Proteolytic activity of *P. proteolytica* WS 5128 was also initially measurable at these time points. However, *aprX* transcripts were already determined earlier in the log-phase, which indicates a regulatory effect on the AprX biosynthesis at the translational or post-translational

level. Our findings correspond with studies determining proteolytic activity in the late exponential and early stationary phase for the first time, independent of the growth temperature (Matselis and Roussis, 1998, Stevenson et al., 2003, Nicodeme et al., 2005, Dufour et al., 2008). According to the literature, an unknown signal associated with high cell densities activates the Gac/Rsm regulation pathway, which then influences the translation of *aprX* (Heeb et al., 2002, Kay et al., 2005, Cheng et al., 2013). It was shown that the lack of *gacA* or *gacS* results in a strongly reduced AprX production and consequently decreased proteolytic activity in *P. protegens* CHA0 and *P. fluorescens* SBW25 (Sacherer et al., 1994, Valverde et al., 2003, Zuber et al., 2003, Cheng et al., 2013).

One possible cell density-associated factor could be calcium. Calcium is essential for the stability of AprX peptidase and it was shown that the addition of calcium to the medium led to an increase in proteolytic activity in *P. fluorescens* and *P. chlororaphis* (Liao and McCallus, 1998, Nicodeme et al., 2005, Ertan et al., 2015, Zhang et al., 2015). In *P. aeruginosa,* calcium is assumed to bind directly to LadS, a sensor kinase that interacts with GacS, important for regulating virulence factors (Broder et al., 2016). Homologs of LadS were also found in *P. syringae* pv. syringae B728a and *P. protegens* CH0A (Humair et al., 2009, Records and Gross, 2010). Since calcium in milk is initially associated with casein micelles and released with increased caseinolytic activity, a correlation between increasing cell density and elevated solvent-calcium concentrations with regard to the activation of the Gac/Rsm pathway could be assumed. However, further investigations are required to confirm this hypothesis.

Moreover, an effect of quorum sensing through N-acyl homoserine lactone (AHL) on the AprX production was discussed controversially in the literature (Liu et al., 2007, Pinto et al., 2010, Martins et al., 2014, Li et al., 2019). Liu et al. (2007) proposed a transcriptional regulation through direct or indirect interaction between AHLs and the *aprX*-promotor in *P. fluorescens* strain 395 (Liu et al., 2007). Further, Li et al. demonstrated increased proteolytic activity in *P. fluorescens* CP032618 depending on the type of AHLs, namely C_4-HSL and C_{14}-HSL led to an elevated caseinolytic activity, while C_{10}-HLS had no influence (Li et al., 2019). In contrast, neither quorum quenching nor supernatants received from bacterial culture nor synthetic AHLs caused variations in the proteolytic activity of raw milk-borne *P. fluorescens* 07A and 041 strains (Pinto et al., 2010, Martins et al., 2014). According to our findings, the mRNA levels of *aprX* were independent from the growth phase. Thus, direct transcriptional regulation of the

AprX biosynthesis in *P. proteolytica* WS 5128 by quorum sensing seems to be very unlikely.

Apart from extrinsic factors, the impact of *aprX*, *prtA*, *prtB,* and *lipA2* on AprX biosynthesis was investigated via respective deletion mutants of *P. proteolytica* WS 5128. Our results regarding the Δ*aprX* mutant confirm data from the literature, assigning the enzyme AprX as the single secreted peptidase with caseinolytic function in *Pseudomonas* (Woods et al., 2001, Maunsell et al., 2006, Maier et al., 2020).

The double deletion mutant strain *P. proteolytica* Δ*prtAB* exhibited wild type-like mRNA levels of *aprX, aprD* and *lipA2* at 12 °C and 30 °C, but the proteolytic activity and the AprX amount were clearly decreased after 48 h and slightly lower after 72 h at 12 °C. Thus, our results indicate that the putative autotransporter proteins PrtA and PrtB might regulate the *aprX-lipA2* operon gene expression at the translational level. However, at 30 °C *P. proteolytica* Δ*prtAB* behaved like wild type. In addition, the results received by the single deletion mutants *P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB* were partly contradictory. For both strains, fewer transcripts of *aprX*, *aprD,* and *lipA2* than for *P. proteolytica* Δ*prtAB* and the wild type were determined after 24 h at 12 °C, and also less caseinolytic activity and AprX amounts were measured after 48 h. This would suggest that *prtA* and *prtB* are either important for transcription regulation of mRNA stability at low temperatures. Interestingly, *P. proteolytica* Δ*prtA*, showed WT-like AprX amounts and proteolytic activity after 72 h at 12 °C, while *P. proteolytica* Δ*prtB* behaved like *P. proteolytica* Δ*prtAB* by revealing less caseinolytic activity than the wild type.

At 30 °C, mRNA amounts were elevated for *P. proteolytica* Δ*prtA* and *P. proteolytica* Δ*prtB* after 24 h, but showed WT-level after extended cultivation, whereas proteolytic activity and AprX amount were always similar to the wild type. Consequently, further experiments, like the complementation of the gene deletions, are required to determine to role of PrtA and PrtB in the AprX biosynthesis.

In *P. proteolytica* WS 5128 the proteins PrtA (1036 aa) and PrtB (985 aa) exhibit an amino acid similarity of 53 %. Both PrtA and PrtB are described as putative autotransporters, homologous to the serine proteases Ssp-h1 (38 % sequence similarity) and Ssp-h2 (39 % sequence similarity) in *Serratia marcescens* (Kawai et al., 1999). As Ssp-h1/h1 are anchored in the outer membrane, PrtA and PrtB are suspected to be also cell-associated (Ohnishi et al., 1997, Kawai et al., 1999). However, PrtA and PrtB were not shown to be caseinolytic (Woods et al., 2001), which

is in agreement with our results of *P. proteolytica* Δ*aprX* and *P. proteolytica* Δ*aprX* Δ*prtAB,* showing the identical phenotype on skim milk agar. Moreover, PrtA and PrtB were assumed to be associated with biofilm formation and cell aggregation (Kawai et al., 1999, Woods et al., 2001, Ma et al., 2003, Sun and Sun, 2015). Thus, PrtA and PrtB could also be a part of a feedback loop in casein degradation or involved as a co-factor in AprX secretion, but this is highly speculative. Although the function and possibly regulatory role of *prtAB* and its gene products in AprX production remains unclear, an influence at the transcriptional level or an effect on mRNA stability is unlikely based on our results.

As the transcription analysis of *aprX*, *aprD*, *prtA* and *prtB* exhibited similar transcription patterns in the wild-type strain, our results confirm previous studies, in which the *aprX-lipA2* operon is described as a polycistronic operon (Woods et al., 2001, McCarthy et al., 2004). In contrast to diverse *aprX* transcription, we noticed no temperature-dependent alterations in the mRNA levels of the lipase genes *lipA2*, located at the operon's distal end, which was also observed by Woods et al. (2001). This strengthens previous studies' results, assuming either an additional promoter located upstream of *lipA2* or another regulatory element, like differing segmental decay rates of the operon's mRNA (Rauhut and Klug, 1999, Woods et al., 2001, McCarthy et al., 2004).
In *P. proteolytica* Δ*lipA2,* transcription levels of aprX were similar to the wild type at 12 °C, which makes an influence of the peptidase on the AprX synthesis rather improbable. In contrast, at 30° C, elevated *aprX* transcripts, increased AprX amounts, and higher proteolytic activity were observed for *P. proteolytica* Δ*lipA2* in comparison to the wild type. Hypothetically, this mighty be due to a regulatory effect associated with mRNA stability by forming secondary structures that enable or disable RNases' access for mRNA degradation. A prediction of such structures or the location of restriction sites might bring exclusion for the *lipA2* function.
Interestingly, the lipase LipA2 in *Pseudomonas* spp. was shown to underlie low-temperature regulation, which leads to a continuous decrease of the lipolytic activity with increasing temperatures (Woods et al., 2001, Rajmohan et al., 2002). However, it is not certain if this effect is based on a temperature-dependent regulation of LipA2 biosynthesis or if the co-secretion with the AprX peptidase could result in the hydrolysis of LipA2 by AprX with increasing temperatures (Duong et al., 1994, Rajmohan et al., 2002). As we did not observe any differences in the *lipA2* transcription level,

independent of the cultivation temperature and duration, at least a transcriptional modulation through temperature seems very unlikely. Besides, in some *Pseudomonas* species, the *aprX-lipA2* operon contains an additional lipase gene (*lipA1*), which is either located upstream of *prtA* (*P. protegens* spp.) or between *prtB* and *lipA2* (*P. chlororaphis* spp.) or apart from the *aprX-lipA2* operon (*P. lactis* spp.) (Maier et al., 2020). In *P. fluorescens* Pf0-1, LipA1 and LipA2 share approx. 52 % sequence similarity (Ma et al., 2003). However, the role of *lipA1* in *aprX-lipA2* expression has not been investigated, yet.

In conclusion, our results revealed insights into the multifactor-regulated transcription of the *aprX-lipA2* operon and synthesis of the peptidase AprX in *P. proteolytica* WS 5128. We demonstrated that temperature below the optimum growth temperature most likely upregulates the expression of the *aprX-lipA2* operon at the transcriptional level or through mRNA stability. Besides, the entry into stationary growth phase seems to influence the peptidase production at the translational level, possibly through the Gac/Rsm cascade. Moreover, the operon genes *prtA*, *prtB* and *lipA2* might possibly impact the AprX biosynthesis at different regulatory levels. Although detailed further analyses are required, this study contributes to the elucidation of the complex regulatory network controlling AprX expression in *Pseudomonas proteolytica*, a frequent contaminant of raw milk with the potential to cause instability of UHT milk products (von Neubeck et al., 2015, Baur et al., 2015a, Stoeckel et al., 2016). By better understanding the favorable conditions for peptidase production, preventive measures could be taken in the future to inhibit or reduce AprX biosynthesis in foods.

5. References

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III General Discussion

In raw milk, several psychrotolerant *Pseudomonas* spp. secrete the heat-stable AprX peptidase, which withstands the UHT treatment and remains partly active in the final product. Thereby, even small amounts of enzymes can significantly reduce the shelf life of fabricated UHT milk and other milk products, causing high financial losses for the dairy industry. Stoeckel et al. described bitterness as the first indication for proteolytic decay, which occurred at least after two months in all analyzed milk samples exhibiting an apparent proteolytic activity ≥0.05 pkat/ml (Stoeckel et al., 2016a). Since the application of long heating times or increased temperatures are more harmful to the end product than beneficial for the inactivation of AprX, removing the peptidase from milk during food processing is nearly impossible (Stoeckel et al., 2016b). Thus, to improve the UHT milk quality, either the AprX production by pseudomonads needs to be reduced in raw milk throughout cold storage, or the peptidase amounts need to be properly assessed in order to adapt downstream product fabrication of contaminated raw milk. Therefore, this project aimed to extend the knowledge of regulatory mechanisms on different levels of the AprX biosynthesis in *Pseudomonas* related to external conditions, such as temperature and growth phase, and internal factors like the presence of selected *aprX-lipA2* operon genes. Moreover, to assess the proteolytic spoilage potential of distinct *Pseudomonas* species on milk products, distribution analysis of the *aprX* locus in the genus *Pseudomonas* was conducted, and the genetic organization of the *aprX-lipA2* operon structure was correlated with the proteolytic activity. Finally, two triplex qPCR assays were established in order to quantify the most proteolytic and common *Pseudomonas* spp. in raw milk.

1 The AprX biosynthesis of *Pseudomonas* spp. is regulated by temperature and growth phase

The production of AprX by pseudomonads is a process that is modulated by a variety of external factors such as iron content (Maunsell et al., 2006), oxygen (Matselis and Roussis, 1998), cultivation temperature (Hellio et al., 1993, Nicodeme et al., 2005) or the medium composition (Nicodeme et al., 2005). Although the underlying regulatory mechanisms are mostly unknown, their understanding is essential to reduce the AprX

production in raw milk in the future. As a consequence, storage, transport, and processing conditions could be adapted to impede the AprX secretion.

1.1 Temperature modulates AprX production on transcriptional level

Temperature is one of the main factors influencing AprX-associated premature milk spoilage. It affects the growth of psychrotolerant pseudomonads, which is highest between 25 °C and 30 °C (Hellio et al., 1993, Nicodeme et al., 2005), and at the same time has an impact on the substrate turnover rates of the peptidase, culminating between 35 °C and 40 °C (Baur et al., 2015b, Glück et al., 2016). Presumably, the latter is why some strains, like *P. chlororaphis CIP 103295* (Nicodeme et al., 2005) and *P. fluorescens* 07A (Alves et al., 2018), exhibit higher proteolytic activity at 30 °C than at 20 °C. However, many others, such as members of *P. chlororaphis* (Nicodeme et al., 2005)*, P. fluorescens* (Hellio et al., 1993, Nicodeme et al., 2005), and *P. proteolytica* WS 5128 (unpublished results of II. chapter 3), were shown to behave vice versa by displaying increased caseinolytic activities at temperatures between 12 °C – 20 °C compared to 30 °C. Consequently, this indicates that the proteolytic potential of *Pseudomonas* spp. is strongly species- and partly strain-specific and that the AprX synthesis must be regulated through a temperature-dependent mechanism. A receptor, which senses these temperature changes and the signal transduction mechanism, is so far unknown.

One possibility might be the temperature-regulated sensor kinase RetS, which was shown to influence the production of antibiotics and hydrogen cyanide at the translational level through interacting with the Gac/Rsm signaling pathway in *Pseudomonas protegens* CHA0 (Humair et al., 2009). The Gac/Rsm pathway is highly conserved in *Pseudomonas* spp. and regulates the cost-intensive synthesis of secondary metabolites and exoenzymes, including the AprX peptidase (Heeb and Haas, 2001). Several studies showed a translational regulation of the AprX biosynthesis for *P. protegens* CHA0 and *P. entomophila* (Blumer et al. 1999, Heeb et al., 2002, Valverde et al., 2003, Kay et al., 2005, Liehl et al., 2006). In a study conducted by Liehl et al. a *gacA* deletion strain of *P. entomophila* was not proteolytic any more*,* and Heeb et al. and Valverde et al. observed that mutations in *gacA* or *gacS* resulted in strong repression of the AprX synthesis in *P. protegens* CHA0 (Heeb et al.,

2002, Valverde et al., 2003, Liehl et al., 2006). In addition, a triple deletion of *rsmX*, *rsmY* and *rsmZ in P. protegens* CHA0 resulted in a decreased AprX production, and the overexpression of RsmA lead to downregulation of the *aprX* translation (Blumer et al., 1999, Kay et al., 2005).

RetS acts as an antagonist to GacS, which inhibits the GacS autophosphorylation, the phosphotransfer to GacA, and probably forms a heterodimer with GacS. Thus, the Gac/Rsm signaling cascade is blocked, and the target genes' translation cannot occur (Goodman et al., 2009, Workentine et al., 2009, Mancl et al., 2019). For *P. protegens* CHA0 it was shown that the interaction of RetS and GacS is much stronger at 35 °C than at 30 °C. Moreover, a point mutation in the *retS* resulted in a temperature-independent expression of the sRNAs RsmX/RsmY/RsmZ (Humair et al., 2009). A RetS homolog (87 % amino acid similarity to the protein of *P. protegens* CHA0) was also identified in *P. proteolytica* WS 5128. However, the wild type and the *retS* deletion mutant strain exhibited similar proteolytic activity when cultivated at 12 °C and 30 °C (unpublished results of II. chapter 3). Consequently, the AprX biosynthesis is most likely modulated independent of RetS at temperatures below 30 °C, at least for *P. proteolytica* WS 5128.

Besides, for *P. proteolytica* WS 5128, generally higher mRNA levels of *aprX* were detected at 12 °C than at 30 °C. Thus, it seems more likely, that temperature either directly modulates the transcription of *aprX* or causes alterations in the mRNA stability (unpublished results of II. chapter 3, figure I9). Burger et al. described a putative two-component system (PrtIR) in *P. fluorescens* LS107d2, which regulates the *aprX* transcription in response to high-temperature stress (Burger et al., 2000). Thereby, *prtR* is located downstream of *prtI*, encoding for a membrane-bound receptor (PrtR) and a putative promotor-binding sigma factor (PrtI). However, the regulation of the AprX biosynthesis through PrtI and PrtR is discussed controversially in the literature (Burger et al., 2000, Okrent et al., 2014). In the study conducted by Burger et al. both, *prtR* and *prtI* were required for *aprX* transcription at 29 °C but were not needed at 23 °C (Burger et al., 2000). In contrast, Okrent et al. did not observe a temperature-dependent effect of PrtIR on the AprX production, neither at 28 °C nor at 20 °C (Okrent et al., 2014). Homologs of PrtI (86 % amino acid similarity) and PrtR (74 % amino acid similarity) from *P. fluorescens* LS107d2 were also identified in *P. proteolytica*.

However, whether PrtI and PrtR participate in the temperature-dependent regulation of the *aprX* transcription in *P. proteolytica* WS 5128 remains unanswered. Thus, the construction of respective deletion strains might clarify their role in *P. proteolytica* WS 5128. Furthermore, in *P. fluorescens* B52 and LS107d2 putative recognition sequences in the *aprX* promotor region might enable the interaction with other ECF-sigma factors or δ^{70} (Burger et al., 2000, Woods et al., 2001). As the *aprX* promotor region of *P. proteolytica* WS 5128 shares 87 % sequence similarity with *P. fluorescens* B52, another temperature-regulated mechanism, modulated through alternative ECF-sigma factors, also seems possible. Therefore, to get the first hint for genetic factors involved in the low temperature-dependent regulation of the *aprX* transcription, a transposon mutagenesis could be performed, selecting strains without temperature-dependent differences in the proteolytic behavior.

1.2 Translational regulation of the *aprX* expression in *P. proteolytica* WS 5128 depends on the growth phase

Another factor that was investigated concerning the influence on AprX synthesis was the cell density. Although high *aprX* transcription levels were determined for *P. proteolytica* WS 5128 in the exponential growth phase, the caseinolytic activity was initially detected in the early stationary phase (unpublished results of II. chapter 3). Similar results were obtained for different members of *P. fluorescens* and for *Pseudomonas* sp. LBSA 1 showing proteolytic activities first after reaching the early stationary phase (Matselis and Roussis, 1998, Nicodeme et al., 2005, Dufour et al., 2008). Therefore, either the proteolytic activity of these strains is below the detection limit in the exponential phase, or most likely the AprX production is regulated at a post-transcriptional level (unpublished results of II. chapter 3). In this context, an influence of the Gac/Rsm signaling cascade on AprX production at the translational level was indicated for *P. protegens* CHA0, as the Gac/Rsm signal transduction system is also initially active in the early stationary phase (Heeb and Haas, 2001). In addition, as mentioned above, point mutations in *gacS* or *gacA* result in a substantial reduction of proteolytic activity in *P. protegens* CHA0 (Heeb et al., 2002, Valverde et al., 2003). However, it has not yet been shown which environmental signal, associated with high cell counts, triggers the Gac/Rsm transduction pathway and whether it interacts directly

with the sensor kinase GacS, or is transmitted indirectly via additional receptors (figure I 9).

One possible signal could be Ca^{2+}, mainly bound to the casein micelles in milk. Nevertheless, through the hydrolysis of casein by the AprX peptidase, Ca^{2+} might be released. In *P. aeruginosa* PAO1, Ca^{2+} was shown to be perceived through the membrane-bound receptor LadS resulting in activation of the Gac/Rsm signaling pathway to switch from acute to chronic host infection (Broder et al., 2016). The conducted study revealed that LadS, after Ca^{2+}-binding, transduces the signal to GacS. Thereby, signal transfer either occurs via physical interaction or through phosphotransfer, resulting in an increased *rsmY* mRNA transcription (Broder et al., 2016). LadS homologs have also been found in several pseudomonads, including *P. protegens* CHA0 (Humair et al., 2009, Workentine et al., 2009), *P. syringae* pv. syringae (Records and Gross, 2010), *P. fluorescens* SBW25, and *P. protegens* Pf-5 (Broder et al., 2016). As, Ca^{2+} did not cause increased *rsmY* transcription in a *P. aeruginosa* PAO1 *ladS* mutant strain, which was complemented with *ladS* of *P. fluorescens* spp. or *P. protegens* spp., Broder et al. assumed that perception of Ca^{2+} by LadS is specific for *P. aeruginosa* (Broder et al., 2016). However, to ensure that Ca^{2+} is not sensed by LadS in milk-associated pseudomonads and not associated with high cell densities, the construction of a *ladS* deletion strain in *P. proteolytica* WS 5128 would be the next reasonable step.

Apart from Ca^{2+}, the effect of quorum sensing (QS) molecules, especially different N-acyl homoserine lactones (AHLs), on *aprX* expression was discussed in previous studies (Liu et al., 2007, Pinto et al., 2010, Martins et al., 2014, Li et al., 2019). QS is applied for cell-cell communication in a wide variety of Gram-positive and Gram-negative bacteria, including *Pseudomonas* spp. to promote, e.g., biofilm formation or secretion of extracellular enzymes, depending on the cell density (Bai and Rai, 2011). Liu et al. assumed a transcriptional effect on *aprX* expression, as the degradation of AHLs resulted in a decreased *aprX*-promotor activity (Liu et al., 2007). Moreover, Li et al. could show that the addition of exogenous AHLs, especially C_4-HSL and C_{14}-HSL resulted in a significant rise in proteolytic activity (Li et al., 2019). However, Pinto et al. and Martins et al. did not observe any effect of AHLs on the proteolytic activity in *P. fluorescens* spp. (Pinto et al., 2010, Martins et al., 2014). If QS regulates the AprX

biosynthesis in some *Pseudomonas* strains of a *Pseudomonas* population, the question arises to what extent less proteolytic pseudomonads can possibly trigger the AprX secretion of ones with high proteolytic potential or vice versa. Thus, supernatants received from less proteolytic species could be added to cultures of high caseinolytic *Pseudomonas* species to analyze the effect on their proteolytic behavior.

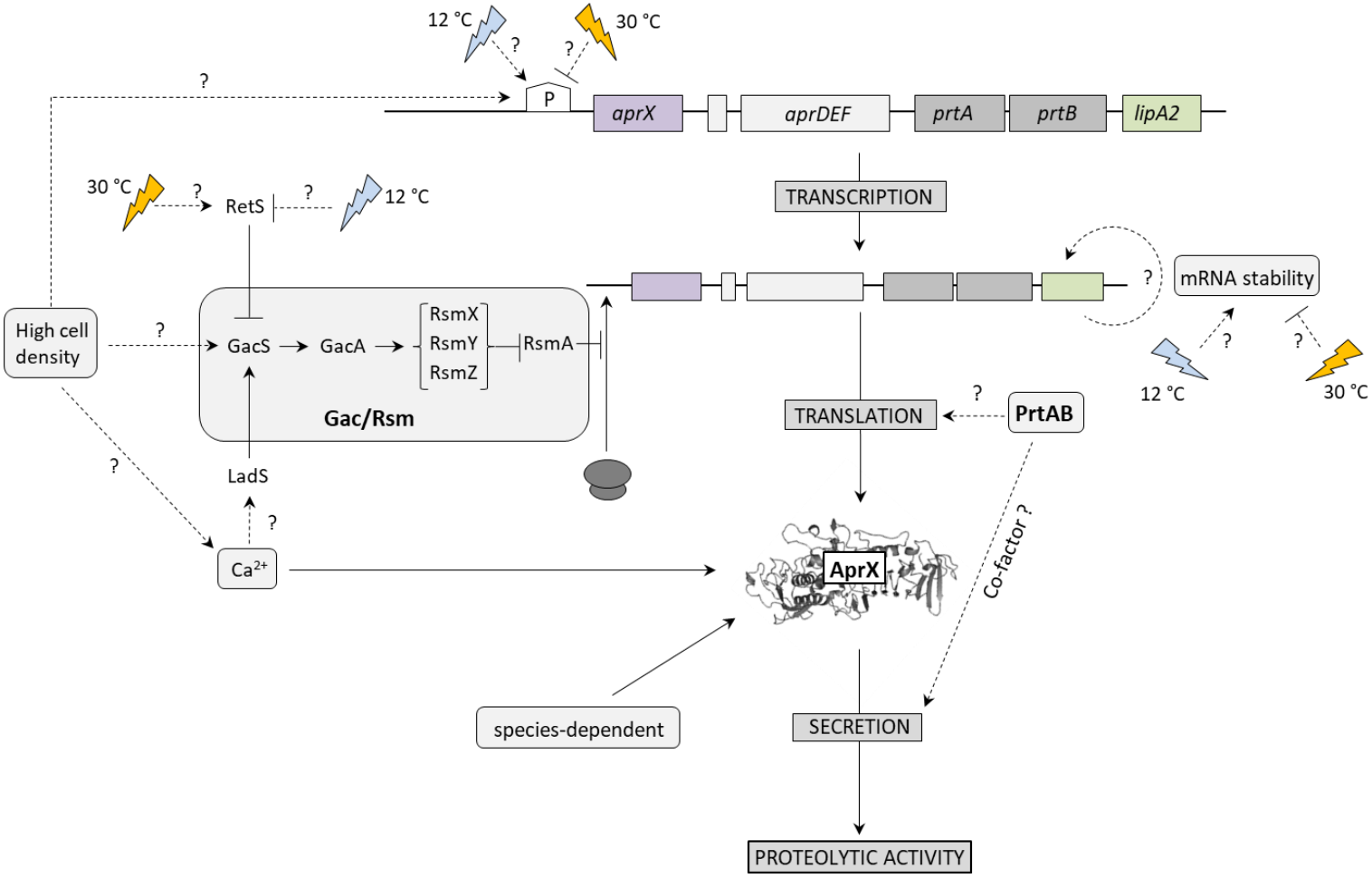

figure I 9: Schematic overview of the regulation of AprX biosynthesis. Positive effects are indicated by arrows (↓), inhibitory effects by a vertical bar (⊥).a distinction is made between confirmed dependencies (solid lines) and possible effects (dashed line) . The figure summarizes findings from Burger et al. 2000, Heeb et al. 2001,2002, Zuber et al. 2003, Kay et al. 2005, Humair et al. 2009, Ertan et al. 2015, Broder et al. 2016, Volk et al. 2019 and unpublished results of II. chapter 3.

2 The genetic organization of *aprX-lipA2* operon indicates the proteolytic potential of *Pseudomonas* spp.

In general, the presence of the *aprX* locus, which was designated to a monophyletic group including 88 different *Pseudomonas* species, is the minimum genetic requirement for the hydrolysis of milk casein (Woods et al., 2001, Liehl et al., 2006, Maier et al., 2020, unpublished results of II. chapter 3). As mentioned above, the level of AprX production in many strains depends largely on environmental parameters such as temperature or growth medium (Nicodeme et al., 2005, Alves et al., 2018, unpublished results of II. chapter 3). Beyond that, it was shown that genetic factors, like the organization of the *aprX-lipA2* operon and the function of selected operon genes, also influence and predefine the caseinolytic potential in *Pseudomonas* spp. (Maier et al., 2020).

The analysis of 129 different *Pseudomonas* strains lead to the identification of 22 distinct structures of the *aprX-lipA2* operon (Maier et al., 2020). Almost all share a conserved part containing five core genes (*aprXIDEF*) and a variable region including up to four different genes (*prtA*, *prtB*, *lipA1*, *lipA2*), which alters in the present and arrangement of the genes (Woods et al., 2001, Ma et al., 2003, Maier et al., 2020). Primarily, species harboring the operon structure of type 1 (*aprXIDEF prtAB lipA2*) or type 9 (*aprXIDEF prtAB lipA2* | *lipA1*) were found to be highly caseinolytic. Both types were solely found in species assigned to the milk-relevant subgroups *P. gessardii* and *P. fluorescens*. In contrast, members of the *P. fragi* subgroup were far less proteolytic, and type 2 (*aprXIDEF lipA2*) and type 8 (*aprXIDEF lipA2* | *prtAB*) were the most abundant structures (Maier et al., 2020). Also, several other studies revealed that representatives of the subgroups *P. gessardii*, e.g., *P. meridian, P. proteolytica*, *P. lactis,* or *P. fluorescens,* were in general highly proteolytic, while those assigned to the *P. fragi* subgroup, such as *P. fragi* or *P. lundensis,* exhibited less activity (Wiedmann et al., 2000, von Neubeck et al., 2015, Caldera et al., 2016, Glück et al., 2016, Maier et al., 2020).

Remarkably, highly proteolytic *Pseudomonas* isolates mainly enclosed *prtA* and *prtB* in the *aprX-lipA2* operon. In addition, strains with the *prtAB* genes located outside the

operon, i.e., type 3 (*aprXIDEF* | *prtAB*) and type 8 (*aprXIDEF lipA2* | *prtAB*), revealed mostly higher proteolytic activities compared to ones lacking *prtAB* completely, like in the operon structure of type 2 (*aprXIDEF lipA2*). Hence, an influence of *prtA* or *prtB* on the caseinolytic activity caused by AprX can be assumed (Maier et al., 2020).

However, the function of PrtA and PrtB has not been clarified yet. Both proteins were described as putative autotransporters (Woods et al., 2001), sharing 53 % amino acid sequence similarity (Kawai et al., 1999, unpublished results of II. chapter 3). Moreover, PrtA and PrtB were shown to be homologous to the serine proteases Ssp-h1 (38 % aa sequence similarity to PrtA) and Ssp-h2 (39 % aa sequence similarity PrtB) found in *Serratia marcescens* (Kawai et al., 1999). Similar to the homologous serine proteases Ssp-h1 and Ssp-h2, the proteins PrtA and PrtB are presumably anchored in the cell membrane and not secreted into the surrounding medium due to structural features, like the lack of the junction region, which is important for protein folding and export (Ohnishi et al., 1997, Kawai et al., 1999). For *Pseudomonas*, it could not be shown that PrtAB itself hydrolyses casein, since *aprX* deletion mutant strains of *P. proteolytica* WS 5128 and *P. fluorescens* B52 were not caseinolytic, though transcription of *prtAB* was comparable to that of the wild types (Woods et al., 2001, Son et al., 2012, unpublished results of II. chapter 3).

Regarding the possible role of PrtAB in AprX synthesis, this study revealed that the proteolytic activity of *P. proteolytica* Δ*prtAB* was approx. one-third reduced in the stationary phase compared to *P. proteolytica* WS 5128 wild type when cultivated in milk at 12 °C. Nevertheless, the *aprX* transcript level of the mutant strain was comparable to the wild type. In addition, this effect was not observed for cultivation at 30 °C. Thus, these findings provide initial evidence that PrtAB may modulate the AprX biosynthesis at the translational level at low temperatures (unpublished results of II. chapter 3, figure I 9). Controversially, the deletion of the single genes, *prtA* or *prtB,* mainly resulted in a reduced (12 °C) or increased (30 °C) *aprX* mRNA level during exponential growth but behaved similarly to the wild type in the stationary phase (unpublished results of II. chapter 3). Consequently, further studies should focus on complementation experiments to investigate whether the observed effects can be reversed.

In the literature, PrtAB was suggested to be involved in biofilm formation as an autoaggregation factor important for adhesion in *Pseudomonas brassicacearum* (Chabeaud et al., 2001). In other bacteria, like seafood-borne *Vibrio parahaemolyticus* strains or *Aeromonas hydrophila* isolated from a clinical context, the biofilm formation and exoenzyme production correlated positively (Khajanchi et al., 2009, Mizan et al., 2016). Besides, Teh et al. demonstrated increased exoprotease production in a biofilm-forming co-culture of *P. fluorescens* C224 and *Staphylococcus aureus* SF01 compared to planktonic cells (Teh et al., 2012). Whether PrtAB plays a role in biofilm formation of *Pseudomonas* spp. and how this is related to the proteolytic behavior has not yet been elucidated. Consequently, in a further study, *prtAB* could be overexpressed from a plasmid in a low proteolytic *Pseudomonas* strain that naturally lacks a *prtAB* locus to monitor variations in the AprX production and the ability to adhere to certain surfaces. If biofilm formation and proteolytic activity are related to the *prtAB* locus, *prtAB* presents a potential target for detection methods such as a qPCR assays, which could be applied to improve the quality of dairy products in the future.

3 Multiplex qPCR assays enable quantification of proteolytic and common *Pseudomonas* species in raw milk

Since UHT-treated milk can be stored at room temperature for up to 6 months, even small amounts of peptidases can cause considerable quality losses. It was shown that defects such as bitterness occasionally occur after already approx. 4 months at a proteolytic activity of ≥ 0.03 pkat/ml (Stoeckel et al., 2016a). One way to assess the peptidase load in milk is through direct detection of the enzyme. Therefore, Volk et al. developed an indirect ELISA assay applying a monoclonal antibody against AprX of *P. lactis*. However, the detection limit was determined around 500 pkat/ml in milk, and the heterogeneity of the AprX sequence within the genus caused a reduced binding affinity of the antibody (Volk et al., 2021a).

Another way to evaluate raw milk quality is to quantify the peptidase-producing pseudomonads. However, each raw milk sample has its unique composition of the *Pseudomonas* population. Whereas species like *P. proteolytica*, *P. lundensis*, *P. lactis*, *P. fragi,* or *P. gessardii* have been isolated frequently from raw milk, others such as *P. paralactis, P. protegens,* or *P. haemolytica* are less abundant (Marchand et al., 2009a,

De Jonghe et al., 2011, von Neubeck et al., 2015, Caldera et al., 2016, Maier et al., 2021). In addition, and as mentioned above, *Pseudomonas* spp. were shown to be highly heterogeneous in their proteolytic potential. In several studies, members of *P. proteolytica* or *P. protegens* exhibited higher proteolytic activities than those assigned to *P. fragi* or *P. lundensis* isolates (von Neubeck et al., 2015, Maier et al., 2020). Consequently, selective cultivation to determine the total *Pseudomonas* counts in milk seems insufficient in order to assess the risk of proteolytic milk decay. Thus, the species-specific detection of common *Pseudomonas* spp. through a multiplex qPCR assay presents an appropriate technique.

So far, only two multiplex qPCR assays have been developed for the detection of *Pseudomonas* species in milk. One method was designed to detect the three species *P. lundensis*, *P. putida*, and *P. fragi* simultaneously by targeting the *carA* gene (Ercolini et al., 2007). The other assay allows the specific identification of *P. fluorescens* through amplifying regions in *adnA* and *fliC* (Xu et al., 2017). However, both assays neglect prevalent species in raw milk with high proteolytic potentials such as *P. proteolytica* or *P. lactis* and do not serve to determine the cell counts. Thus, in this study two triplex qPCR assays to quantify the most common *Pseudomonas* spp. in raw milk and differentiate between species with high (*P. proteolytica*, *P. gessardii*, *P. lactis,* and *P. protegens*) and moderate-low proteolytic potential (*P. lundensis* and *P. fragi*), was developed. Thereby the high sequence variability of the *aprX* gene served for the design of five species-specific hydrolysis probes. Moreover, another probe targeting a conserved region in *rpoB* to enumerate the total *Pseudomonas* counts was established (Maier et al., 2021).

Depending on the respective probe, a linear detection range between approx. 10^3–10^7 cfu/ml and minimal quantifiable counts of 2×10^2–2×10^3 cfu/ml were received (Maier et al., 2021). Other qPCR assays, especially ones to enumerate human pathogens, like *Salmonella* spp., *B. cereus*, *L. monocytogenes,* and *S. aureus,* often apply a pre-enrichment step to receive detection limits below 10^2 cfu/ml (Forghani et al., 2016, Heymans et al., 2018, Parichehr et al., 2019). However, for our qPCR assay, the implementation of such a time-consuming pre-treatment was not necessary, as the amount of pseudomonads in raw milk usually lies between 10^2 to 10^5 cfu/ml (Leriche

and Fayolle, 2012, von Neubeck et al., 2015, Skeie et al., 2019), which is covered by the obtained detection range (Maier et al., 2021).

Besides the adequate sensitivity, all species-specific hydrolysis probes of the qPCR turned out to be highly specific. The only false-positive signals were received for isolates phylogenetically closely related to the target species (Maier et al., 2021). However, as these species were rarely isolated from raw milk (von Neubeck et al., 2015, Maier et al., 2020) and mostly proteolytic, their incorrect detection by the qPCR assay can be tolerated (von Neubeck et al., 2015, Maier et al., 2020, Maier et al., 2021). Furthermore, all milk-relevant *Pseudomonas* spp., including *P. proteolytica*, *P. lundensis*, *P. lactis, P. gessardii,* and *P. fragi* (von Neubeck et al., 2015, Caldera et al., 2016), were successfully detected via the universal *Pseudomonas* probe (Maier et al., 2020). Only very weak signals were generated for 5 out of 40 other bacteria tested, including *Pseudoalteromonas haloplanktis* WS 5482 and *Citrobacter freundii* WS 5466 with the universal *Pseudomonas* probe (Maier et al., 2020). *Pseudoalteromonas* spp. are halophilic marine bacteria, which were occasionally found on the surface of soft smear-ripened and semi-hard cheese or in cheese rinds (Feurer et al., 2004, Quigley et al., 2012, Almeida et al., 2014, Zhang et al., 2016, Salazar et al., 2018). Moreover, *Citrobacter* spp. were associated with β-lactamase production, but Odenthal et al. isolated only 10 representatives by analyzing 866 different raw milk samples, and Geser et al. did not identify any member of *Citrobacter* in 100 milk samples tested (Geser et al., 2012, Odenthal et al., 2016). Consequently, the few received false-positive signals did not significantly reduce the assay's high specificity.

In addition, the universal *Pseudomonas* hydrolysis probe was utilized in order to determine total *Pseudomonas* counts in milk samples. Remarkably, the results were in good agreement with cell counts from cultivation, however, cell counts enumerated on CFC-agar were partly elevated (Maier et al., 2021). This effect might be due to a lack of selectivity for the latter medium, e.g., for several Enterobacteriaceae, *Acinetobacter* spp., *Aeromonas* spp. and *Serratia* spp. (Flint and Hartley, 1996, Salvat et al., 1997, Yu et al., 2019), which were commonly or occasionally found in raw milk (Sørhaug and Stepaniak, 1997, von Neubeck et al., 2015, Baur et al., 2015b, Breitenwieser et al., 2020). In summary, the developed triplex qPCR assays present a

promising method to improve milk quality and assess the spoilage potential depending on the composition of the *Pseudomonas* population in the future. Compared to current methods, it is less time-consuming (3 h) and far more specific, which is why it is already an improvement (Maier et al., 2021). Nevertheless, extensive studies still need to be conducted to relate cell counts and AprX load in raw milk to define thresholds to evaluate the milk quality and adapt further processing.

4 Conclusion

The AprX peptidase production through psychrotolerant *Pseudomonas* species underlies several external and internal factors, which enormously complicate the assessment of the enzyme load in raw milk. In this project, temperature and growth phase were shown to modulate the AprX biosynthesis at different levels. At 12 °C, significantly higher mRNA levels were observed than at 30 °C, indicating an up-regulation of AprX production on transcriptional level by lower temperature. Moreover, proteolytic activity was first detected when cells reached the stationary phase, although *aprX*-transcription was already measured in the exponential phase. Thus, an additional regulation of the AprX biosynthesis at the translational level via the Gac/Rsm signaling cascade is highly probable. Nevertheless, the signal associated with high cell counts has not yet been identified, but Ca^{2+} and QS have been discussed. Consequently, the storage time between milking and downstream processing should be reduced to a minimum to prevent excessive cell growth and enzyme secretion of *Pseudomonas* spp., which later can reduce UHT milk's shelf life.

Furthermore, this study demonstrated that the *aprX* gene is the minimum requirement for caseinolytic potential, and it could be assigned to a monophyletic clade within the genus *Pseudomonas*. Also, milk-related species were primarily allocated to the subgroups *P. gessardii*, *P. fluorescens,* and *P. fragi*. Whereas members of the subgroups *P. gessardii* and *P. fluorescens* exhibit primarily high caseinolytic potential, ones of *P. fragi* showed moderate or low activity. These differences could be attributed to variations in the *aprX-lipA2* operon structure, especially to the presence and location of *prtAB*. However, the structure of the *aprX-lipA2* operon is extraordinarily heterologous in *Pseudomonas* spp. and partly even differs between closely related

species. This illustrated that the enumeration of the total *Pseudomonas* counts in raw milk is insufficient to assess its quality regarding the proteolytic potential.

Finally, two triplex qPCR assays to quantify the most common *Pseudomonas* spp. in raw milk according to their proteolytic potential were successfully established. Thus, a highly sensitive application, which is less time-consuming and more specific than selective cultivation, was developed. Still, further work is required to define appropriate thresholds and correlate critical *Pseudomonas* cell counts with problematic AprX amounts or enzyme activities. Consequently, the triplex qPCR assay could help assess the AprX load in raw milk in the future, and the downstream fabrication of milk products could be adapted accordingly. For example, milk products with a short storage time could be made from raw milk, revealing a high enzyme load, while UHT milk and milk products would only be produced from low-contaminated raw milk.

IV References

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Oral presentations

„AprA peptidase production of *Pseudomonas* isolates from raw milk“, VAAM Jahrestagung 2018, Wolfsburg, Germany, April 2017

„Entwicklung eines spezies-spezifischen qPCR Assays zum Nachweis von Pseudomonaden in Rohmilch“, Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising, Germany, October 2019

„Entwicklung eines spezies-spezifischen Multiplex qPCR Assays zur Risikoabschätzung von proteolytischem Milchverderb durch Pseudomonaden“, 18. Fachsymposium Lebensmittelmikrobiologie, Kiel, Germany, October 2019

Poster presentations

„AprA peptidase production of *Pseudomonas* spp. isolated from raw milk”, 16th International Conference on *Pseudomonas*, Liverpool, UK, September 2017

„Development of a multiplex qPCR assay for detection of proteolytic *Pseudomonas* species in raw milk”, FEMS2019 – 8th Congress of European Microbiologists, Glasgow, UK, July 2019

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EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die bei TUM School of Life Sciences der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Quantification of *Pseudomonas* spp. in raw milk and molecular analysis of their proteolytic potential

am Lehrstuhl für Mikrobielle Ökologie unter der Anleitung und Betreuung durch Herrn Prof. Dr. Siegfried Scherer ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Ab. 6 und 7 Satz 2 angebotenen Hilfsmittel benutzt habe.

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Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich einverstanden.

Ort, Datum, Unterschrift

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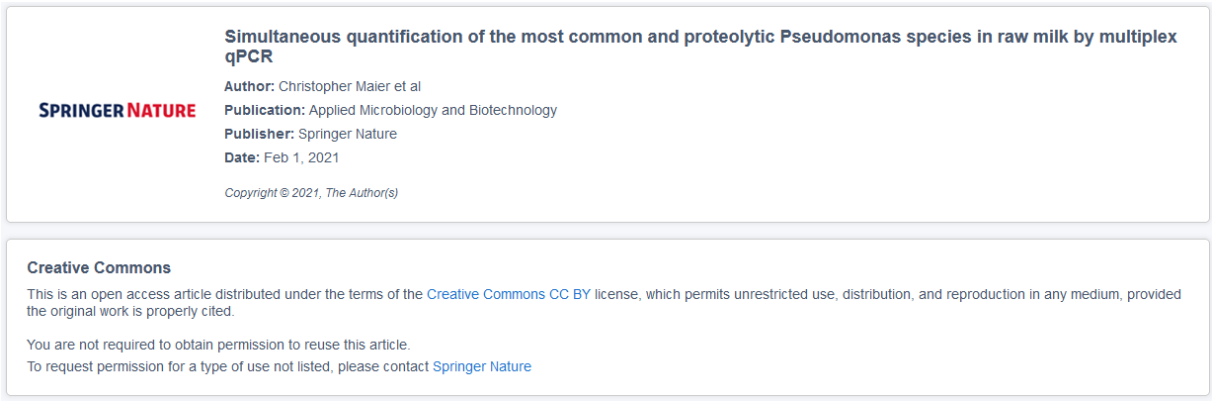

SPRINGER NATURE

Simultaneous quantification of the most common and proteolytic Pseudomonas species in raw milk by multiplex qPCR

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