Dissertation

Comparison of *Bacillus cereus sensu lato* from patient, industrial and food packaging samples, its antimicrobial susceptibility and sliding motility as interfering phenotype in AST

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

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Disclosures

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Abbreviations

AST	antimicrobial susceptibility testing
CIP	ciprofloxacin
CLI	clindamycin
Col-S	Columbia agar with 5% sheep blood
ERY	erythromycin
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.
GO	gene ontology
IMI	imipenem
LEV	levofloxacin
LIN	linezolid
MALDI-TOF MS	matrix-assisted laser desorption ionization–time of flight mass spectrometry
MER	meropenem
MH	Mueller Hinton
MHS	Mueller Hinton swarming agar
MIC	minimal inhibitory concentration
MLST	multi-locus sequence typing
MRSA	methicillin resistant *Staphylococcus aureus*
OD600	optical density at a wavelength of 600 nm
TrA	TrA swarming medium (trypton, NaCl, agar-agar)
VAN	vancomycin

Abstract

The ubiquitous *Bacillus cereus* group or *Bacillus cereus sensu lato* (s.l.) comprises several closely related species with manifold impacts on humans. In a clinical context, *Bacillus cereus* s.l. is primarily known as a causing agent of self-limiting food poisoning. However, non-gastrointestinal infections with *Bacillus cereus* s.l. may require rapid and targeted anti-infective therapy as these infections can be life-threatening. The aim of this dissertation was therefore to dissect the presence and antimicrobial susceptibility of *Bacillus cereus* s.l. in different patient samples and environmental, human-associated samples. A total of 353 isolates from patient samples, cranial orthoses, food packaging materials and industrial water and biofilm samples were compared. The isolates from routine patient diagnostics showed the most even distribution of phylogenetic groups, indicating a random origin of the bacterial strains. In contrast, isolates from a clinical trial on cranial orthoses and the corresponding hospital environment indicated potential nosocomial origin. In addition, the patient isolates accumulated more antimicrobial resistances to clinically relevant antibiotics than environmental isolates from food packaging materials. Using the disk diffusion method, the most frequent resistant phenotypes among all isolates were reported for erythromycin, carbapenems and vancomycin, while linezolid, clindamycin, and fluoroquinolones yielded high susceptibility rates. Since putative vancomycin resistance has hardly been studied in the *Bacillus cereus* group, the subsequent focus was on vancomycin-resistant isolates. The resistance observed in agar diffusion tests could be disproved by utilizing both broth microdilution and agar dilution. Moreover, microscopic imaging revealed a motility strategy of *Bacillus cereus* s.l. to undermine correct susceptibility testing for vancomycin. Using a food packaging isolate as a model, sliding motility was discovered as the mechanism responsible for the translocation into the inhibition zone during agar diffusion testing. RNA-Seq analysis on the Oxford Nanopore platform confirmed the sliding motility at the gene expression level. The expression of the biofilm machinery was increased in sliding cells, while virulence genes were repressed. In addition, swarming motility could be effectively distinguished from sliding motility, both phenotypically and at the gene expression level. Susceptibility testing based on agar diffusion was shown to be unreliable for vancomycin due to sliding motility, which should be included in relevant guidelines. Future research should also endeavor to elucidate whether this distinct motility may similarly impact the susceptibility testing of other antibiotics including erythromycin and carbapenems.

Zusammenfassung

Die ubiquitäre *Bacillus cereus* Gruppe oder *Bacillus cereus sensu lato* (s.l.) besteht aus zahlreichen sehr eng verwandten Spezies mit unterschiedlichen Auswirkungen für den Menschen. Im klinischen Kontext ist die *Bacillus cereus* Gruppe hauptsächlich als Erreger selbstlimitierenden Lebensmittelvergiftungen bekannt. Jedoch können ihre Vertreter auch lebensbedrohliche non-gastrointestinale Infektionen versuchen, die eine rasche und zielgerichtete antibiotische Therapie verlangen. Ziel dieser Dissertation war es daher das Vorhandensein und die Empfindlichkeit gegenüber Antibiotika von *Bacillus cereus* s.l. in Proben von Patient*innen zu charakterisieren und mit Isolaten aus human-assoziierten Umweltproben zu vergleichen. Dafür wurden 353 Isolate aus der mikrobiologischen Routinediagnostik von Patient*innen, einer klinischen Studie zu Helmorthesen und der zugehörigen Krankenhausumgebung, Lebensmittelverpackungsmaterialen und industriellen Wasser- und Biofilmen untersucht. Die Isolate von Patient*innen zeigten die gleichmäßigste Verteilung an phylogenetischen Gruppen, was auf einen zufälligen Ursprung der Isolate schließen lässt. In Kontrast dazu lässt sich ein nosokomialer Ursprung der Isolate von Helmorthesen und Krankhausumgebung annehmen. Die Isolate von Patient*innen wiesen in Hemmhoftests mehr Antibiotikaresistenzen als die Umweltisolate auf, darunter besonders Resistenzen gegenüber Erythromycin, Carbapeneme und Vancomycin. Für Linezolid, Fluorochinolone und Clindamycin konnte eine hohe Empfindlichkeit beobachtet werden. Im Anschluss wurde der Fokus auf die bisher kaum erforschte Vancomycin-Resistenz in *Bacillus cereus* s.l. gelegt. Mittels Mikrodilution und Agardilution konnte die zuvor vermutete Vancomycin-Resistenz widerlegt werden. Darüber hinaus konnte anhand eines Isolats aus einer Lebensmittelverpackung mikroskopisch eine „sliding motility“ als verantwortlicher Mechanismus identifiziert werden, welcher zum Wachstum im Hemmhof bei Vancomycin führt. Die Ergebnisse der RNA-Seq-Analyse bestätigten diese Beobachtung auf Ebene der Genexpression. Die „sliding motility“-Zellen exprimierten besonders Gene der Biofilmmaschinerie, während die Expression der Virulenzgene unterdrückt wurden. Zudem konnte eine effektive Unterscheidung von „sliding motility“ und Schwärmen sowohl phänotypisch als auch auf Ebene der Genexpression erzielt werden. Durch „sliding motility“ führt die Resistenztestung von Vancomycin durch Agardiffusionstests zu unzuverlässigen Ergebnissen, was in relevanten Richtlinien vermerkt werden sollte. Ob „sliding motility“ auch die Testung von Erythromycin und den Carbapenemen beeinflusst, muss noch beantwortet werden.

Introduction

Bacillus cereus – one group with multiple implications

Bacillus cereus is one of the most complex species constructs in the field of microbiology and therefore challenging and fascinating microbiologists throughout the sub-disciplines of their field. Its relevance covers the industrial and agricultural sectors, and the insecticidal properties discovered in distinct strains built a cornerstone in the development of genetically modified crops. On the contrary, several strains pose a threat to consumers, as food poisonings and food contamination caused by *Bacillus cereus* are still one of the biggest issues in food safety. In clinical microbiology, *Bacillus cereus* can be associated with a potential bioweapon (anthrax) and the highest safety measures on the one hand, but it also has the status of a neglected pathogen on the other hand, which is often hard to distinguish from sample contamination. During the past decades, numerous unique species and strains were discovered within the framework of *Bacillus cereus*. However, the genetic backgrounds of *Bacillus cereus* strains in various situations are so closely related that they have become a textbook example for complex and confusing taxonomy and phylogenetics. Therefore, due to the multiplicity of phenotypes, the phylogenetic proximity and the broad relevance, microbiologists nowadays prefer the terms *Bacillus cereus* group or *Bacillus cereus sensu lato* (s.l.), which aim to reflect both the strong homogeneity and the variability of the *Bacillus cereus* species. However, these terms are also informal and traditional species nomenclature is still in use.

The Latin name *Bacillus* means stick or rod, while *cereus* is derived from the Latin word *cera*, which means wax, and can be translated as wax-like. Members of the *Bacillus cereus* group share several phenotypical characteristics. They are Gram positive and endospore-forming bacteria with a typical rod shaped morphology and a rather large size of approximately 1.0 to 1.2 µm in width and 3.0 to 5.0 µm in length. (1) The majority of species within *Bacillus cereus sensu lato* are mesophilic and prefer moderate growth temperatures ranging from 10 to 40° C. However, psychrophilic and thermophilic strains or species are also part of the species complex. (2) All members of *Bacillus cereus sensu lato* are usually catalase positive, non-fastidious, aerobic or facultatively anaerobic. Swarming and swimming motility has been shown for many species within the *Bacillus cereus* group except for the non-motile *Bacillus anthracis*. (3)

Bacillus cereus sensu lato is regarded as a ubiquitous group of species with the soil environment being the predominant habitat. Strains were isolated from a plethora of diverse

environments including different plant tissues, aquatic environments such as rivers, rainwater and groundwater, marine environments, dust and different foods. (4–6) Furthermore, several members of the *Bacillus cereus* group are commensal inhabitants of the intestinal microbiota of animals and humans. (1,4) *Bacillus anthracis,* the causative agent of anthrax, is particularly associated with livestock and the carcasses of infected animals, where it can persist for extended periods (7). The ubiquitous occurrence of *Bacillus cereus sensu lato* and its potential to cause opportunistic infections necessitate the acquisition of up-to-date knowledge about its antimicrobial susceptibility, not only in patient-associated strains but also in the environment. Therefore, this work summarizes the taxonomic framework of the *Bacillus cereus* group, its virulence factors, and their relevance in different environments (clinical diagnostics, food packaging, and industrial biofilms). Furthermore, it provides evidence of antimicrobial susceptibility by comparing patient isolates and environmental strains, with food packaging and industrial isolates serving as examples.

Bacillus cereus sensu lato – the formation of a species complex

The complex taxonomy of *Bacillus cereus sensu lato* originally consisted of four species: *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides*, which were identified in the late 19th and early 20th century. (8) *Bacillus cereus* or *Bacillus cereus sensu stricto* has been regarded as a food poisoning species capable of producing potent toxins that cause diarrhea and emesis. The molecular etiology of diarrheal and emetic food poisoning could be elucidated to a large extent with several different enterotoxins and the emetic toxin cereulide as major virulence factors. (9) *Bacillus anthracis* is the causing agent of anthrax, a potentially lethal zoonotic disease associated with herbivores including sheep and cattle. The wildtype *Bacillus anthracis* is also considered a potential bioweapon that may require biosafety level 3 measures. (10) Compared to other *Bacillus cereus sensu lato* species, *Bacillus anthracis* is typically non-motile, hemolysis-deficient and susceptible to penicillin. (3) In the pre-sequencing era, these characteristics were routinely used to distinguish *Bacillus anthracis* strains in both clinical and veterinary diagnostics. *Bacillus thuringiensis* is characterized by its insecticidal properties arising from the delta endotoxins (δ-endotoxins). This large group of toxins is also called crystal (Cry or Cyt) toxins or Bt toxins and are lethal to the larvae of primarily Lepidoptera, Diptera or Coleoptera. (11) Nowadays, *Bacillus thuringiensis* and its most economically significant strains, *B. thuringiensis* var. *israelensis* (Bti) and *B. thruingiensis* var. *kurstaki* (Btk),

as well as their purified toxins have a multitude of applications in agriculture. These include pest control on crops and the management of disease vectors such as mosquitos in urban areas. (12) The toxin itself played an important role in the development of transgenic crops, when initially the *crylA(b)* gene was incorporated into maize (*Zea mays*) leading to the synthesis of insecticidal proteins in the plant tissue. (13) *Bacillus mycoides* was phenotypically distinguished by its colony shape and rhizoid growth pattern, which displayed root-like structures and spreading on solid growth media. (8)

Since 1998, the number of novel species assigned to *Bacillus cereus sensu lato* has drastically increased. Initially, the psychrotolerant *Bacillus weihenstephanensis*, which grows at temperatures as low as 4 °C, and the rhizoid *Bacillus pseudomycoides*, which showed a colony morphology similar to *Bacillus mycoides*, emerged and were then followed by the proposal of numerous other novel species. (14,15) *Bacillus cytotoxicus* was published as a high-risk species defined by the presence of the potent *cytK-2* variant of the Cytotoxin K gene responsible for severe gastrointestinal syndromes. (16) Moreover, *Bacillus cytotoxicus* is considered thermotolerant with a maximum growth temperature of 50 °C. In contrast, *Bacillus toyonensis*, which was proposed as novel species in 2013, was intended to be used as the probiotic supplement Toyocerin® in livestock breeding. (17) However, the approval of as probiotic supplement was suspended due to safety concerns and the potential spread of antibiotic resistances. (18)

The rapid increase in *Bacillus cereus sensu lato* species gave rise to a problem in defining correct criteria for the species definition. The initial classification was based on the phenotypical characteristics of the *Bacillus cereus* group species such as the synthesis of distinct toxins (*Bacillus thuringiensis*) or the colony shape (*Bacillus mycoides*), as well as on the clinical characteristics (*Bacillus anthracis, Bacillus cereus s.s.*). Given that a significant proportion of toxins in *Bacillus cereus sensu lato* is encoded on plasmids, (10) the species defining properties were based on mobile genetic elements, which are prone to horizontal gene transfer. Both the anthrax toxin and the poly-γ-D-glutamate capsule, which are essential virulence factors for *Bacillus anthracis*, are encoded on the pXO1 (182 kb) and PXO2 (96 kb) virulence plasmids, respectively. (10) In fact, the anthrax toxin genes containing pXO1 plasmid was described in non-*anthracis Bacillus cereus* isolates causing anthrax-like disease in patients, although the strains were phenotypically and biochemically identified as *Bacillus cereus* (motility, beta-hemolysis). (19,20) Furthermore, *Bacillus anthracis* capsule encoding genes were identified on

plasmids of other *Bacillus* species. (21) In an insect infection model, Yuan et al. were able to transfer the crystal toxin encoding plasmid pHT73 of *Bacillus thuringiensis* to six other *Bacillus cereus* group strains by conjugation. (22) Following the dogma of crystal toxins as species marker for *Bacillus thuringiensis*, conjugative transfer or loss of plasmids could change entire species affiliations. The emetic *Bacillus cereus sensu stricto* causing severe food poisonings was initially defined by the synthesis of the emetic toxin cereuilde. The cereuilde encoding plasmid, however, has also been found in psychrotolerant *Bacillus weihenstephanensis,* (23) and was recently detected in a *Bacillus mobilis* strain (also called *Bacillus mosaicus*). (24) In addition, enterotoxin genes responsible for diarrheal food poisoning are prevalent in the entire *Bacillus cereus* group regardless of the presence of other plasmid-encoded toxin genes. The species affiliation based on certain phenotypical characteristics or the presence of single genes, therefore, was not only ignoring paradigms in actual phylogeny based on the genetic evolution of species and strains but also resulted in a volatile definition of a taxonomic species. Furthermore, standard approaches in bacterial identification such as 16S rRNA gene sequencing are not applicable for *Bacillus cereus sensu lato*, as sequence similarity of the 16S rDNA usually exceeds 99% among the species. (25,26) Consequently, it has also been suggested that the *Bacillus cereus* group only represents lineages or subspecies of a single species, *Bacillus cereus*, rather than comprising distinct species. (27)

The propagation of whole genome sequencing (WGS) allowed a phylogenetic approach that took actual genetic evolution rather than morphology or mobile genetic elements into account. As a result, several novel species were published based on genetic relationships and the number of genomes submitted to genomic databases increased. At the same time, however, the risk of misinterpretation and taxonomic confusion reached a new level. Liu et al. (6) published the identification of nine novel species (*Bacillus albus, B. luti, B. mobilis, B. nitratireducens, B. pacificus, B. paramycoides, B. paranthracis, B. proteolyticus, B. tropicus*) based on WGS and in 2022, a review article on current *Bacillus cereus* phylogeny listed 23 proposed and published species within the *Bacillus cereus* group. (8) As illustrated very well by Carroll et al., (8) the lack of strict guidelines in the WGS era significantly affects the reliability of novel species proposals. Nowadays, species affiliations are usually based on average nucleotide identities (ANI) giving a percentage value of genomic identity between two species. Since the threshold of identity for defining a novel species is not strictly regulated, species affiliations can vary according to the applied threshold. In addition, the field of phylogenetics is permanently subject of re-structuring

and re-classification. With regard to *Bacillus cereus sensu lato*, even the basis of the taxonomic order has changed as the phylum of Firmicutes, which comprised the family of Bacillaceae and the genus of *Bacillus* contained therein, has recently been renamed to Bacillota. (28) Another aspect of an increasingly complex conglomerate of species names is potential misidentification, which has already happened for *Bacillus paranthracis* and *Bacillus anthracis* in food poisoning events. (29)

In the past, several attempts were made to resolve the increasingly complex taxonomy within *Bacillus cereus sensu lato* aiming to provide both accurate phylogenetic classification and a framework for clinical and economical risk assessment. For this purpose, molecular typing schemes were established, based on different genes or gene sets. Single locus sequence typing using either *groEL, gyrB*, *panC, rpoB* were designed to cluster strains based on the evolution of single housekeeping or virulence genes, (30–34) while multi-locus sequence typing (MLST) combines sequence information of up to seven genes enabling the assignment to sequence types and clonal complexes. (35–37) Beyond traditional MLST, core genome MLST (cgMLST) schemes were developed for *Bacillus anthracis* and *Bacillus cereus sensu lato* based on 3,803 and 1,568 core genes, respectively. (38,39) The cgMLST is a standardized, high-resolution typing scheme for detailed strain characterization. However, it is labor and cost intensive, given that WGS data is required.

The most popular single locus sequence typing based on the *panC* gene sequence is derived from a previous MLST scheme. (35) Due to its simple approach combined with fast analysis and sufficient phylogenetic resolution of the major lineages of *Bacillus cereus sensu lato*, it is still the most widespread phylogenetic method. The *panC* gene is involved in pantothenate biosynthesis and part of the cofactor coenzyme A biosynthesis pathway. It codes for a pantoate-beta-alanine ligase, which catalyzes the ATP-dependent condensation of (R)-pantoate and beta-alanine to AMP, diphosphate and (R)-pantothenate. (40,41) Using 425 independent strains from various sources including food poisoning events and reference strains, Guinebretière et al. (31) developed the typing scheme and divided *Bacillus cereus sensu lato* into seven phylogenetic groups, each containing distinct species and phenotypical traits. The *panC* classification also allows a basic categorization according to pathogenicity and the clinical significance of strains. (2) The initial proposal of the phylogenetic *panC* classification defines *Bacillus pseudomycoides* strains as group I, containing only mesophilic and low virulent strains. Group II includes strains of *Bacillus cereus* and *Bacillus thuringiensis* with mesophilic growth and food poisoning

potential. *Bacillus anthracis* forms group III together with strains of *Bacillus cereus* and *Bacillus thuringiensis*. Originally, cereulide-producing strains were also exclusively detected in group III. The *panC* groups IV and V contain mesophilic strains of *Bacillus cereus* and *Bacillus thuringiensis*. The type strains *Bacillus cereus sensu stricto* ATCC 14579 and *Bacillus thuringiensis* serovar Berliner strain ATCC 10792 were assigned to group IV. The *panC* group VI contains *Bacillus mycoides* and *Bacillus thuringiensis* strains as well as psychrotolerant *Bacillus weihenstephanensis*. The *panC* group VII only comprises the highly virulent *Bacillus cytoxcius*. In addition to the major phylogenetic clades, subgroups within each *panC* group have been identified and associated with certain properties. Following the *panC* typing scheme, clinical significance is highest in group III due to high enterotoxin production, potential cereulide production and the presence of *Bacillus anthracis* strains. The food poisoning risk remains high for groups VII, IV, decreases with group V, and is low for group VI. (2,31) Conversely, Drewnowska et al. (42) detected the highest in vitro cytotoxicity within the groups II and IV. Compared to previous species definitions, the *panC* typing scheme provides both phylogenetic classification and basic information about clinical importance and therefore, it has routinely been used in the description of isolates from food poisoning outbreaks, non-gastrointestinal infections, food contamination or environmental background.

Recently, the *Bacillus cereus sensu lato* complex has been restructured to incorporate detailed phylogenetic information based on WGS as well as clinically and economically important phenotypes. This genomospecies/subspecies/biovar nomenclature is currently the most comprehensive framework for *Bacillus cereus sensu lato* and includes eight published genomospecies: *Bacillus pseudomycoides*, *Bacillus paramycoides*, *Bacillus mosaicus*, *Bacillus cereus sensu stricto*, *Bacillus toyonensis*, *Bacillus mycoides*, *Bacillus cytotoxicus* and *Bacillus luti*. (43) Additionally, the four putative genomospecies *Bacillus bingmayongensis*, *Bacillus gaemokensis*, *Bacillus clarus* and *Bacillus manliponensis* are included. The *Bacillus mosaicus* subspecies *anthracis* and subspecies *cereus* were introduced to cover the clinically important clonal lineages of *Bacillus anthracis* and emetic *Bacillus cereus*, which are now incorporated into *Bacillus mosaicus*. Furthermore, the concept of biovars (e.g. *Bacillus mycoides* biovar Emeticus, previously known as emetic *Bacillus weihenstephanensis*) takes the phenotypical or molecular detection of distinguishing toxins into account, such as anthrax toxin, cereulide or Bt toxins. As a result, well-established species names like *Bacillus thuringiensis* are no longer in existence but were transformed into biovars. In accordance with the taxonomic changes proposed by Carroll et

al, (44) the seven-group scheme was also adjusted to an eight-group framework, which is largely congruent with the genomospecies nomenclature. The commonly used PubMLST seven-gene MLST scheme can also be used for genomospecies assignment. Therefore, the genomospecies/subspecies/biovar nomenclature currently is the most universal framework to classify *Bacillus cereus sensu lato* strains either using WGS data, *panC* group assignment, MLST sequence types, molecular data about virulence genes or phenotypical information such as toxin synthesis.

Table 1: Phylogenetic groups of *Bacillus cereus sensu lato* based on the *panC* gene sequences.

Adjusted eight-group *panC* typing (44)	**Seven-group *panC* typing** (31)	**genomospecies nomenclature of *Bacillus cereus sensu lato*** (43)
I	I	*Bacillus pseudomycoides*
II	II, III, V*	*Bacillus mosaicus, Bacillus luti*
III	III	*Bacillus mosaicus*
IV	IV	*Bacillus cereus sensu stricto*
V	V	*Bacillus toyonensis*
VI	VI	*Bacillus mycoides, Bacillus paramycoides*
VII	VII	*Bacillus cytotoxicus*
VIII	II, III, VI	*Bacillus mycoides*

* *panC* sequence identities of these strains were below 80% with the reference sequence

Virulence factors of *Bacillus cereus sensu lato*

Among the multitude of different bacteria within the *Bacillus* genus, the *Bacillus cereus* group comprises the clinically most relevant species. This group has evolved a set of diverse strategies to facilitate niche colonization, nutrient acquisition, resistance to exogenous stressors and the dissemination to new environments. Although species classified as *Bacillus cereus sensu lato* are closely related phylogenetically, different forms of pathogenicity have evolved. Some of the key virulence factors are shared on transmissible plasmids, while others are located on the chromosome and follow a strictly vertical transmission. (45) In general, the toxin spectrum of

Bacillus cereus sensu lato includes exotoxins, which are secreted to damage host tissue or evade immune responses, and endotoxins, such as the insecticidal Cry toxins of *Bacillus thuringiensis* or biovar Thuringiensis. (9,11) Motility and multicellular behavior such as biofilms enable species within *Bacillus cereus sensu lato* to colonize new hosts and to resist eradication efforts. (46,47) A set of antibiotic resistance genes contributes to increased persistence in host tissue. (48)

The following section presents and discusses the essential virulence factors of non-*anthracis Bacillus cereus sensu lato*, which play an important role in food poisoning and non-gastrointestinal infections, as well as in the persistence and spread during food processing, industrial manufacturing, and healthcare facilities. The distinctive pathogenicity of *Bacillus anthracis* or biovar Anthracis will not be elaborated in depth, as the impact of anthrax disease and the extent of pathogenicity differ considerably from those of the other species. The pathogenicity of *Bacillus anthracis* basically relies on two key virulence factors: the poly-γ-D-glutamate capsule and the anthrax toxin, which consists of three proteins called protective antigen (PA), lethal factor (LF) and edema factor (EF). (49) Due to its incisive importance as a foodborne pathogen, previous research on virulence factors of *Bacillus cereus sensu lato* has been focused on the implications in food poisoning events rather than in non-gastrointestinal infections. Despite their cytotoxic potential, the actual role of many enterotoxins and cereulide in non-gastrointestinal infections remains still unclear. In contrast to the pivotal impact of singular virulence factors, such as those observed in anthrax disease, a combination of multiple factors, is believed to shape infections with *Bacillus cereus sensu lato.* (50)

The most important enterotoxins comprise the non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl) and cytotoxin K (CytK). (9) The non-hemolytic enterotoxin is a three-component toxin encoded in the *nheABC* operon. Contrary to its name, this pore-forming enterotoxin shares structural properties with other hemolysins and exhibits hemolytic activity against erythrocytes of various species as well as cytotoxic effects against Vero and primary endothelial cells. (51,52) Additionally, the pore formation induces the inflammasome and stimulates inflammatory pathways in macrophages. (53) The non-hemolytic enterotoxin appears to be the most prevalent enterotoxin among *Bacillus cereus sensu lato* strains and was detected in approximately 85%–100% of all strains. (9,54) Hemolysin BL is also a three-component enterotoxin, encoded in the *hblDAC(B)* operon, which leads to the formation of hetero-oligomeric pores in the membranes of erythrocytes and various other target cells. (9) Early studies with rabbit ileal loops demonstrated

the cooperative effects of the single toxin components causing a massive fluid accumulation in the ileum. (55) In addition, hemolysin BL has been demonstrated to exhibit dermonecrotic and cytotoxic effects, as well as to increase vascular permeability. (55,56) Together with the non-hemolytic enterotoxin, the synergistic effects of hemolysin BL on the induction of the inflammasome and inflammation-mediated cell death were observed. (53) In comparison to the *nheABC* operon, the *hblDAC(B)* genes are less prevalent and are present in 40%–70% of the strains. (9) It is assumed, however, that both operons have evolved from the common ancestor gene as sequence identities have been detected between the components of the two toxins. (45)

In addition to the three-component enterotoxins, Cytotoxin K is a single protein, which forms oligomeric pores associated with diarrheal food poisoning. Two variants of the *cytK* gene, called *cytK-1* and *cytK-2*, have been described, which show a high degree of sequence homology, but differ significantly in their pathogenic potential. (57) Approximately 40%–70% of *Bacillus cereus sensu lato* strains carry the *cytK-2* variant, while the significantly more toxic variant encoded by *cytK-1* is exceptionally present in the rare *Bacillus cytotoxicus.* (9) According to the proposed genomospecies nomenclature, *Bacillus cytotoxicus* marks a unique genomospecies and is the only representative of the *panC* group VII. (2,44) The role of CytK-2 during diarrheal food poisoning has been questioned despite the demonstration of hemolytic and cytotoxic activity. (57,58) In contrast, CytK-1 exhibited higher cytotoxic activity compared to CytK-2 and has been associated with severe cases of food poisoning and bloody diarrhea. (57,59) The cytotoxic effects also included inflammasome activation resulting in a highly inflammatory form of cell death. (60) Similar to the *hblDAC(B)* operon, *cytK* genes underwent frequent events of horizontal gene transfer and deletions. (45)

A poorly characterized enterotoxin, designated as enterotoxin FM (*entFM*), has been identified in a wide range of *Bacillus cereus sensu lato* isolates. (9) However, the actual role of this enterotoxin in the pathogenesis of disease remains uncertain. (61)

The emetic toxin cereulide is a small dodecadepsipeptide that bears a close resemblance to the antibiotic valinomycin and is synthesized by a non-ribosomal peptide synthetase. (62,63) It exhibits high stability and resistance to low and high pH levels, as well as to cleavage by trypsin and pepsin. (64) Consequently, it will not be inactivated during the gastric passage. Cereulide is the key virulence factor for emetic food poisoning and is present in a rare subset of strains

compared to other *Bacillus cereus sensu lato* strains. (9) Phylogenetically, cereulide-producing strains are associated with the *panC* groups III and VI and the genomospecies of *Bacillus mosaicus* subsp. *cereus* and *Bacillus mycoides.* (2,43,44) The concept of subspecies *cereus* and biovar Emeticus account for the medical importance of this toxin, which is responsible for severe and occasionally fatal food poisoning events. In contrast to chromosomally located enterotoxins, the cereulide-encoding *ces* locus (24kb) is located on the pCER270 mega-plasmid, which resembles the pXO1 plasmid carrying the anthrax toxin genes. This *ces* locus consists of seven genes involved in synthesis, regulation and transport of the depsipetide. (63) Moreover, recent studies have demonstrated that cereulide is synthesized along with a cocktail of different isocereulides, which synergistically contribute to the actual cytotoxicity. (65–67) Early studies in *Suncus murinus* have already indicated that cereulide stimulates the vagus nerves via the 5-HT3 serotonin receptors and thereby leading to emesis and nausea via the vomiting center in the medulla oblongata. (62) The emetic stimulus can be so massive as to lead to life-threatening conditions such as Boerhaave syndrome. (68) Beyond stimulation of vomiting, cereulide exhibits high-grade cytotoxicity and disseminates to spleen, liver, brain and fatty tissue via the bloodstream and the blood-brain-barrier. (69) On the molecular level, cereulide is a potassium ion binding toxin with high ionophoric activity. The ionophoric activity is thought to disturb the mitochondrial membrane potential due to increased influx of potassium ions via the outer membrane to the inner membrane. The resulting depolarization leads to mitochondrial swelling and impairs mitochondrial respiration and beta-oxidation of fatty acids. Mitochondrial function is particularly impaired in the liver resulting in acute hepatic failure. (70–72)

The enterotoxins and emetic toxin are the primary determinants of food poisoning events. They are accompanied by a multitude of virulence factors that contribute to non-gastrointestinal infections. In addition to the hemolytic enterotoxins, *Bacillus cereus sensu lato* possesses three hemolysins: hemolysin I (also called cereolysin O, anthrolysin O, or thuringolysin O), hemolysin II and hemolysin III. (73) Hemolysin I belongs to the cholesterol dependent cytolysin family, which also includes perfringolysin O, streptolysin O and listeriolysin O from *Clostridium perfringens*, *Streptococcus pyogenes* and *Listeria monocytogenes*, respectively. (73) It is a pore-forming protein that exhibits high toxicity against mammalian cells and induces cell death via pyroptosis, ROS (reactive oxygen species) accumulation, and potassium efflux. (74) Hemolysin II (HlyII) is a hemolytic and beta-barrel pore-forming toxin, which forms pores in various eukarytic

cells and induces apoptosis in macrophages. (75) Hemolysin III has been proposed as another hemolytic protein but has not been purified yet. (73)

In addition to toxins, degradative enzymes are widespread in the *Bacillus cereus* group and are believed to be relevant virulence factors. Among the various lipases and proteases, the phosphatidylcholine- and the phosphatidylinositol-specific phospholipase C enzymes are likely to be involved in pathogenesis due to their membranolytic effects. (76,77) As with phospholipases, the *Bacillus cereus* sphingomyelinase is thought to play a significant role in the pathogenesis of infections (e.g. sepsis). (78) Sphingomylinases are sphingomyelin-degrading enzymes that have destructive effects on eukaryotic cell membranes and are considered potent virulence factors in many pathogens. Furthermore, collagenases of *Bacillus cereus sensu lato* are capable of degrading collagenous tissue. (79,80) The collagenase ColQ1, which was discovered in *Bacillus cereus* strain Q1, is a highly efficient metalloprotease and can profoundly degrade dermal collagen, which resulted in increased fragility of dermal tissue. (81) A number of other metalloproteases, including InhA1, InhA2, InhA2, and NprA, have been the subject of extensive study with regard to their role in pathogenicity. (82) For example, InhA1 and NprA facilitate the bacterial escape from macrophages and degrade a broad range of extracellular tissue such as fibronectin and collagen. (83) Hence, the large set of collagenases and metalloproteases present in strains of *Bacillus cereus sensu lato* is likely to facilitate the dissemination and propagation in host tissue during infection.

A decisive virulence factor of *Bacillus anthracis* or *Bacillus mosaicus* subsp. *anthracis* or biovar Anthracis is the poly-γ-D-glutamate capsule that protects the cells from host defense, particularly from phagocytosis by macrophages. (84) Although the capsule genes (*capBCADE*) of *Bacillus anthracis* have not been detected in other *Bacillus cereus sensu lato* strains so far, encapsulated *Bacillus cereus* have been associated with severe or fatal of pneumonia. (85,86)

At the multicellular level, the ability to form biofilms enables *Bacillus cereus sensu lato* to withstand unfavorable conditions in vivo and in the environment. Biofilms are multicellular structures of bacteria that embed themselves in a self-produced extracellular matrix to form thin layers at the interfaces of liquid and biotic or abiotic surfaces. The principal components of the extracellular matrices are exopolysaccharides, proteins, lipids and nucleic acids. (87) In the *Bacillus cereus* group, several exopolysaccharides and their corresponding loci have been identified, which contribute to biofilm formation e.g. an exopolysaccharide consisting of two

amino sugars, GlcNAcA (N-Acetylglucosamine) and XylNAc (2-N-acetylamino-1,2,4-trideoxy-1,4-iminoxylitol), a Pel-like polysaccharide originally discovered in the Gram-negative *Pseudomonas aeruginosa*, and the *eps1* and *eps2* loci. (88) Two major structural proteins are TasA and CalY, which form extracellular fibrils within the biofilm. (89,90) The putative enterotoxin entFM has been proposed as potential cell wall peptidase involved in adhesion and biofilm formation. (61) The advantages of bacterial biofilms include increased resistance against stress conditions such as antibiotic exposure, desiccation or shear forces. (87) Additionally, they can facilitate coordinated nutrient supply and the possible interchange of mobile genetic elements. (91) Biofilm formation of *Bacillus cereus sensu lato* has been associated with catheter-related bloodstream infections (92,93) and orthopedic implant-related infection. (94)

Bacillus cereus sensu lato has also proven to be an efficient swarming species. (47) Swarming motility is multicellular translocation behavior using flagella to actively spread over surfaces. In addition to the colonization of ecological niches, collective migration such as swarming motility can also increase the infiltration of host tissue and it has been demonstrated that increased secretion of enterotoxins (Hemolysin BL) accompanies swarming behavior in *Bacillus cereus sensu lato*. (95)

In the clinical context, the most relevant feature of the majority of *Bacillus cereus sensu lato* strains is the expression of effective beta-lactamases. (48) Consequently, penicillin and cephalosporin antibiotics are usually ineffective for the treatment of severe infections. The *Bacillus cereus* group is notable for the presence of three distinct beta-lactamases, including one metallo-beta-lactamase, which have been described to mediate intrinsic resistance. The beta-lactamase I is a class A enzyme that is almost completely secreted. (96) The beta-lactamase II (BcII) is a secreted metallo-beta-lactamase of class B with a zinc ion dependent activity. (97) The third beta-lactamase, beta-lactamase III, has been demonstrated to exhibit partial membrane-bound lipoprotein activity and belongs to the class A enzymes. (98) Torkar and Bedenić (48) identified *bla*II genes in all 66 isolates included in their study. Furthermore, they detected $bla_{\text{CTX-M}}$ and bla_{VIM} family genes in several isolates, which led them to conclude that also extended-spectrum β-lactamase and carbapenemase genes are present in *Bacillus cereus sensu lato*. In the past, reports of beta-lactam resistant *Bacillus anthracis* increased, (99) although its susceptibility to penicillin and cephalosporins has been taken as a species defining criterion. (3)

The regulation of virulence gene expression is mainly dependent on the quorum sensing regulator PlcR (Phospholipase C Regulator), which governs the expression of the major enterotoxins (non-hemolytic enterotoxin, hemolysin BL, cytotoxin K) and cereolysin O via binding to the palindromic PlcR-boxes in the promotor region of the respective genes. The small regulatory peptide PapR, which is secreted, processed and reimported by *Bacillus cereus sensu lato* cells, is required for activation of PlcR. In total, the PlcR-PapR system involves at least 45 genes of various functions. (100,101) The *plcR* locus in *Bacillus anthracis* or *Bacillus mosaicus* subsp. *anthracis* is silenced due to a nonsense mutation. (102) As a result, virulence genes regulated by PlcR are expressed only at very low levels, but the anthrax-causing virulence factors on the pXO1 and pXO2 plasmids remain unaffected.

In contrast to most virulence factors, the expression of cereulide encoding genes is independent from the pleiotropic regulator PlcR. The expression of the *ces* gene cluster is tightly orchestrated and regulated by the global regulators CodY and AbrB. (103) In addition, auto-regulatory circuits of individual *ces* genes as well as environmental factors such as temperature, pH, salt concentrations, availability of water or certain nutrients may play crucial roles in cereulide synthesis. (9,104) For example, Kranzler et al. (66) showed that low temperatures are associated with the synthesis of more toxic isocereulides compared to moderate temperatures.

Other virulence factors that are independent from PlcR include the hemolysin II (HlyII) and the metalloproteases InhA1 and NprA. (10) In fact, several other regulatory proteins in *Bacillus cereus sensu lato* are involved in virulence gene expression e.g. the metabolic regulator CodY, the two component-system ResDE, catabolite repression associated CcpA or the phase-transition regulator SinR, the specific regulator HlyIIR, and the global regulator Fur. (9) Many of these factors might contribute concomitantly to the expression of toxins, degradative enzymes and cell modifications. Consequently, the actual regulation and the fine adjustment of virulence gene expression may vary from strain to strain, due to the diversity within the *Bacillus cereus* group.

In summary, the molecular setup of *Bacillus cereus sensu lato* is clearly oriented to colonize animal tissue as an ecological niche rather than a having a solely soil-dwelling saprophytic lifestyle like other *Bacillus* species e.g. *Bacillus subtilis*.

Bacillus cereus as a foodborne pathogen

In 2022, the European Food Safety Authority (EFSA) reported 308 food-borne outbreaks in European countries with 3,216 human cases, 66 hospitalizations and two deaths caused by *Bacillus cereus* toxins. It was the fifth most prevalent causative agent exceeding the number of reported cases caused by *Clostridium perfringens*, *Staphylococcus aureus* and *Campylobacter*. Given that the vast majority of 16,765 cases was of unknown origin, the estimated number of undetected cases may be considerably higher. (105) In general, foodborne diseases caused by *Bacillus cereus sensu lato* can be divided into diarrheal and emetic food poisoning. (1) The diarrheal type of food poisoning is caused by different enterotoxins, which are chromosomally located and widespread among *Bacillus cereus* s.l. strains. (9) The typical route of transmission is the ingestion of food products contaminated with spores of *Bacillus cereus sensu lato*. Since only the acid-resistant endospores survive the gastric passage, they can reach the small intestines, where they germinate to vegetative cells and secrete a set of different enterotoxins. (106) Consequently, the diarrheal type of food poisoning is considered toxicoinfection. (1) The incubation time is 8 to 16 hours after ingestion of spores and symptoms usually last 12 to 24 hours. The symptoms of diarrheal food poisoning include watery diarrhea, abdominal cramps and pain. Nausea may occur but emesis is generally rare. In contrast, emetic food poisoning is caused by the ingestion of preformed cereulide, an extremely acid- and heat-stable emetic toxin. The emetic syndrome is therefore considered a food intoxication, which is characterized by severe emesis, nausea and malaise. (1) Since preformed toxin is consumed, the typical onset of symptoms is after 0.5 to 5 hours post-ingestion, which is considerably faster than for the toxicoinfection. As cereulide-producing strains usually possess genes for the synthesis of different enterotoxins as well, a combined form may be observed, when both preformed cereulide and spores are ingested. (1) The minimal infective dose varies greatly due to the different forms of food poisoning and the different pathogenic potential of strains. A dose of 10^5 to 10^8 colony-forming units per gram (CFU/g) is thought to cause diarrheal food poisoning. (107) As the emetic syndrome is caused by preformed cereulide, the actual amount of toxin present in the food is more important than the number bacterial cells. A minimal intoxication dose of 8 to 10 µg per kg body weight was proposed by the EFSA, (107) but cases of emetic food poisoning could be linked to minimal amounts of 0.01 to 1.25 µg/g food. (108) In general, it is thought that 10^5 to 10^8 CFU/g are also sufficient to produce critical amounts of cereulide in food. (107) Both diarrheal and emetic food poisoning are typically self-limiting diseases, however, severe

outbreaks and cereulide intoxications have also resulted in fatalities and critically ill patients in the past. Emetic food poisoning in particular may lead to severe intoxications mostly attributed to incorrectly stored food. (109) Fatal outcomes of emetic intoxications were associated with the ingestion of incorrectly preserved pasta salad and spaghetti with tomato sauce. (110,111) A massive uptake of cereulide may cause multi-organ failure including acute liver failure, rhabdomyolysis and disseminated intravascular coagulation. (112) Numerous other foodborne outbreaks were reported worldwide irrespective of the geographical region and the type of contaminated food. (109) The number of affected individuals ranged from one person to more than 300. As with other forms of food poisoning, those of a younger age and the elderly are at high risk for developing severe food poisoning syndromes caused by *Bacillus cereus sensu lato*. Therefore, educational facilities, nursery homes and medical facilities should be particularly aware of the pathogenic potential. Since *Bacillus cereus sensu lato* and its resistant spores cannot be avoided in food products, appropriate food storage is essential. This includes temperatures below 5 °C for cold foods and above 57 °C for hot foods to prevent germination of spores and subsequent growth of vegetative cells. Furthermore, leftovers should be rapidly refrigerated below 5 °C as gradual cooling of food over an extended period allows spore germination and growth. Special attention should also be paid to the thawing of frozen food. Since the emetic toxin cereulide can hardly be removed from food once it has formed, reheating is usually not sufficient to inactivate the toxin. A higher prevalence of subtypes such as biovar Emeticus in certain types of food is a topic of intense discussion but has not yet been resolved. (109)

Bacillus cereus in food and food packaging

Bacillus cereus sensu lato has been detected in a multitude of different food products from farm to fork. It has been isolated from fresh vegetables from retail markets including cucumbers, carrots, herbs and salad leaves, fresh meat and meat products, fish and seafood, rice, flour, spices, milk and dairy products. (113–117) Furthermore, high prevalence of *Bacillus cereus sensu lato* has been reported for ready-to-eat foods such as ready-to-eat mixed salad leaves, bakery products, sandwiches, ice cream, cooked rice and noodles or infant formula. (113,118–121) Quantitative assessments of *Bacillus cereus sensu lato* revealed bacterial counts up to more than 3 $\log_{10}$ CFU/g. (122) Ready-to-eat products present an elevated risk, as they are typically not or insufficiently heated prior to consumption and bacterial contamination is therefore

not eliminated. Although the number of *Bacillus cereus sensu lato* in food products is generally below the dose necessary for food poisoning, inadequate storage may lead to bacterial proliferation. Psychrotolerant growth of some strains is a major concern in the food industry and represents a significant challenge for food manufacturers, storage, food logistics, wholesale and retail markets. Carroll et al. (8) even discussed a biovar Weihenstephanensis to account for the risk of psychrotolerant strains in the food-processing sector. Furthermore, the capacity of forming highly resistant endospores often prevents efficient elimination of contaminations by pasteurization during food processing. (104) The emetic toxin cereulide is almost impossible to remove from food products. Due to its size, filtration and bactofugation are ineffective. In the majority of cases, thermal inactivation is not applicable as the toxin is stable for 80 minutes at 121 °C and for 60 minutes at 150 °C at pH 9.5, thereby even exceeding the heat and pH stability of endospores. (123) The hydrophobic properties of both cereulide and bacterial endospores also facilitate their attachment to surfaces in food processing systems. *Bacillus cereus sensu lato* has outstanding ability to adhere to stainless steel surfaces used in the food processing industry, and piping systems with dead legs and residual liquids are particularly susceptible to biofilm formation. (124,125) The attachment of spores can subsequently lead to biofilm formation, which further contributes to bacterial persistence due to increased resistance against cleaning regimes. (126)

Despite high hygienic standards in food processing, contamination with *Bacillus cereus sensu lato* often appears to be inevitable due to its ubiquitous presence. Consequently, routine quality control is essential to maintain high standards in food safety. The primary pathway for contamination of food products, whether processed or native, remains the soil, as it is the natural habitat of *Bacillus* species. (4,104) Nevertheless, *Bacillus cereus sensu lato* is also frequently present in other food-associated environments, such as the food packaging. Paper and cardboard have been used as food packaging material ever since to protect food from chemical, physical or microbiological changes to extend the shelf life and thereby ensure the quality of the food as well as preserve the consumer’s health. Nevertheless, it has long been known that paper and cardboard provide a reservoir for a variety of bacterial and fungal species. (127–130) The manufacturing process of paper and cardboard involves the use of naturally derived raw materials, most importantly cellulose fibers derived from wood, several additives that favor microbial growth, large quantities of water, and a warm to hot environment. Despite the use biocides to control microbial growth in paper machines, both the formation of biofilms and the

presence of bacteria within the final products are inevitable. (131) The sources of bacterial contamination are manifold: fresh water, soil and dust particles associated with wood or carried by air, and the additives used in papermaking. (132,133) Thus, the biofilms associated with a paper machine comprise a high diversity of bacteria including *Bacillus cereus sensu lato* (127,134). The detrimental effects of these biofilms, called biofouling, are major issues in many industrial processes and the biofouling properties of *Bacillus cereus sensu lato* have been emphasized in the corrosion of steel pipelines. (135) In the paper industry, biofouling causes clogging of filters and pipes, sheet breaks and holes. Another consequence of biofilms is the deposit of slime on paper sheets resulting in bacterial contamination of paper and cardboard. (136) The final steps of paper manufacturing include intensive processing at high pressure and high temperatures resulting in an almost exclusive presence of endospore-forming bacteria. (127,137) There have only been few reports on the detection of bacterial species belonging to biological risk group 2, and these are almost exclusively limited to *Bacillus cereus sensu lato*. In a study from 2021, Schmid et al. (138) isolated 75 members of the *Bacillus cereus* group from various fiber-based food packaging materials and analyzed their virulence. In total, diarrheal as well as emetic strains were detected among the packaging material isolates, which were mostly mesophilic. These results highlight the broad distribution of the *Bacillus cereus* group in the food packaging sector. Pirttijarvi et al. (139) characterized bacterial contaminants from liquid packaging boards and reported 22 *Bacillus cereus* group strains. Of these *Bacillus cereus* group isolates, ten could grow at 6 °C confirming the presence of psychrophilic strains in food packaging paper and board. Although neither standard procedures for the assessment of *Bacillus cereus sensu lato* in paper and cardboard nor official limits for microbial counts are published, legal frameworks ensure the microbiological integrity of food packaging paper and cardboard. A benchmark-setting regulation of the European Parliament formulates in Article 3 that materials intended to come into contact with food do not transfer their constituents to food in quantities, which could endanger human health or lead to an unacceptable change in the composition of the food or bring about a deterioration in the organoleptic characteristics thereof. (140) This is corroborated by a study conducted by Ekman et al., (141) which demonstrated that only 0.001 to 0.03% of *Bacillus thuringiensis* spores in food packaging paper are transferable to food.

In summary, the effects of *Bacillus cereus sensu lato* on the food sector are felt from farm to fork and have both industrial and health-related impact. The consumption of food and the use of

packaging materials such as paper and cardboard may actually be the most common ways in which *Bacillus cereus sensu lato* is encountered.

Bacillus cereus in the clinical context – an overlooked pathogen

Its standing as a dreadful agent of food poisonings is in stark contrast to the neglected relevance of *Bacillus cereus sensu lato* as an opportunistic pathogen. While the ingestion of endospores or preformed toxins lead to gastrointestinal diseases, the dissemination of *Bacillus cereus sensu lato* in other regions of the body can cause severe infections that may be life threatening. A variety of local or systemic infections have been reported in both immunocompetent and immunosuppressed patients including cutaneous and soft tissue infections, endophthalmitis, respiratory infections, osteomyelitis, endocarditis, central nervous system (CNS) infections and bloodstream infections. (50,142,143) Despite the low prevalence of non-gastrointestinal infections with *Bacillus cereus sensu lato*, they can be difficult to treat and often have a fatal outcome. In general, immunosuppression and malignant neoplasms are the most critical risk factors for severe infections with the *Bacillus cereus* group. Sepsis and CNS infections in patients with hematological malignancies showed an extremely high mortality (>66%) despite aggressive treatment with broad-spectrum anti-infectives. (144–146) Additional factors contributing to *Bacillus cereus* infections are surgical or traumatic wounds, indwelling catheters, intravenous drug abuse and (preterm) neonates. (142,147) While patients with preexisting comorbidities are at elevated risk, it is important to note that healthy and immunocompetent individuals can also develop fatal infections due to *Bacillus cereus sensu lato*. The reports on fatal or life-threatening infections in immunocompetent individuals included fulminant pneumonia, bacteremia, sepsis, and multiple organ failure. (148) Furthermore, cases of soft-tissue and cutaneous infections have been documented. (149,150) In ophthalmology, *Bacillus cereus sensu lato* is responsible for endophthalmitis and subsequent vitrectomy, accounting for 15 to 43% of infections after ocular trauma. (50,151) It is evident that in immunocompetent individuals there is still a risk for severe infections with *Bacillus cereus sensu lato*. Some infections were linked to previous symptoms of gastroenteritis and suspected food poisonings, which suggests a potential transition from gastrointestinal to generalized infection. (148) Moreover, the ubiquitous occurrence of *Bacillus cereus sensu lato* presents a risk of contaminating wounds, yet also presents a challenge for hospital hygiene, for instance, during surgical procedures. In fact, a nosocomial origin of infections with *Bacillus cereus sensu lato*

could be shown more than once. In 2011, an outbreak was linked to contaminated hospital linen, resulting in several cases of bacteremia. (152) A study conducted by Glasset et al. (153) demonstrated the intra- and inter-hospital contamination with identical *Bacillus cereus* strains, which have been attributed to severe diseases such as sepsis in neonates. However, due to its stigmatization as a frequent sample contaminant, (154) the true burden as an opportunistic pathogen is often underrated. While pseudo-outbreaks by environmental *Bacillus* spp. contributed to the assumption of being a sample contaminant, (155–157) the study by Glasset et al. (153) reported that in 38% of infections *Bacillus cereus* was wrongly considered as a contaminant.

In the field of microbiological patient diagnostics, the identification of *Bacillus cereus sensu lato* typically relies on culturing methods. As a non-fastidious organism, it can be cultivated using basic culture media, including blood agar or tryptic soy agar. Furthermore, several media that are more selective are available to isolate and further differentiate the *Bacillus cereus* group from other *Bacillus* species. Among these, the mannitol egg yolk polymyxin agar (MYP) and different chromogenic agar are commonly used. (158) Additionally, biochemical confirmation can be done in accordance with the BAM protocol, which also provides a protocol for differentiating the clinically most relevant representative, *Bacillus anthracis* or *Bacillus mosaicus* subsp. *anthracis*. (3) To date, identification of *Bacillus cereus sensu lato* is routinely performed via the analysis of protein spectra obtained by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). However, a precise discrimination between the (genomo)species is often not feasible and the levels of species resolution vary between manufacturers. (147) An alternative approach is 16S rRNA gene sequencing, which can provide comparable results regarding the *Bacillus cereus* group. Nevertheless, identifying the *Bacillus cereus* group is often sufficient for empirical treatment and further analyses, such as toxin confirmation and antimicrobial susceptibility testing (AST). For eventual outbreak analysis, molecular methods with higher resolution, such as MLST, cgMLST, and WGS are required.

The treatments of food poisoning and non-gastrointestinal infections differ significantly, as the former are typically self-limiting diseases. Therefore, clinical interventions are rare and only required in severe cases such as extreme dehydration, hemorrhagic events or organ failure due to massive cereulide intoxication. In contrast, non-gastrointestinal infections may require rapid antibiotic treatment and, in some cases, intensive care monitoring. As a consequence of the effective beta-lactamases that are frequently detected in *Bacillus cereus sensu lato*, strains are

typically resistant to penicillin G and aminopenicillin, including ampicillin, ampicillin-sulbactam, amoxicillin, and amoxicillin-clavulanic acid as well as to cephalosporins from first to fourth generation. (48,159) Therefore, the antibiotic therapy typically consists of glycopeptides, carbapenems, fluoroquinolones, lincosamides, aminoglycosides or a combination of these. (144,147). As of today, there is no universal guideline regarding the antibiotic therapy of *Bacillus cereus* infections, however, retrospective studies and comprehensive literature reviews suggest vancomycin as the agent of choice to treat severe and invasive infections with members of the Bacillus cereus group. (50,147,160) Vancomycin is a glycopeptide originally synthesized by *Amycolatopsis orientalis* (*Streptomyces orientalis*), which efficiently inhibits the crosslinking of the N-acetylglucosamine and N-acetylmuramic acid by blocking the D-alanyl-D-alanine residues. (161) Vancomycin is a last-line antimicrobial agent frequently utilized for the treatment of complex infections, such as those caused by methicillin-resistant *Staphylococcus aureus* (MRSA). It is also regarded as an essential medicine by the World Health Organization but vancomycin resistance is an emerging concern in enterococci. (162) Similarly, the glycopeptide teicoplanin has also been demonstrated to be effective in the treatment of infections caused by *Bacillus cereus*, as evidenced by both in vitro results and case reports. (144,147) Both vancomycin and teicoplanin show poor oral bioavailability and therefore require intravenous administration. (163) The second class of important antimicrobials are carbapenems such as imipenem and meropenem, which belong to the beta-lactam antibiotics. Carbapenems are broad-spectrum antibiotics that impair cell wall biosynthesis via the inactivation of the transpeptidases (penicillin-binding proteins) necessary for the crosslinking of peptidoglycan. (164) The beta-lactamases present in *Bacillus cereus* s.l. strains usually have no effect on carbapenems, making them a frequent choice for the treatment. (48,147) In general, carbapenem resistance is a rare but increasing problem due to the global dissemination of carbapenem-hydrolyzing enzymes (carbapenemases). Fluoroquinolones are antimicrobial agents that interfere with DNA replication as they inhibit the bacterial type II topoisomerases. In *Bacillus cereus sensu lato* strains, high susceptibility to ciprofloxacin and levofloxacin has been reported and fluoroquinolones were shown to be effective in the treatment of pneumonia, cutaneous infections, wound infections and bacteremia. (147,149,165,166) Furthermore, lincosamides (e.g., clindamycin) and macrolides (e.g., erythromycin) are often considered for the treatment, as the respective susceptibility rates are estimated to be 75% and 74%. (50,147) Both antimicrobial agents target the protein biosynthesis by inhibiting translation via the large subunit of bacterial ribosomes. However, the widespread use of macrolides and related

substances also harbors the potential for the emergence of resistance, and the similar mechanism of action can result in cross-resistance to macrolides and lincosamides. Other antibiotics that are used against infections with *Bacillus cereus sensu lato* include linezolid and aminoglycosides, (146,147,160) which both target the small ribosomal subunit. However, due to the occurrence of adverse side effects, the latter is often considered as a drug of last resort for serious infections, (167) or it is combined with other antimicrobials such as vancomycin. (144,147)

Acquired resistances in the *Bacillus cereus* group are a relatively rare phenomenon and thus have received minimal investigation. Consequently, the molecular mechanisms underlying the eventually detected resistance phenotypes remain largely undiscovered. Reports of typically unexpected, if not acquired, resistances comprise erythromycin, clindamycin, ciprofloxacin and levofloxacin, but also the clinically rarely used tetracycline, chloramphenicol and rifampicin. (114,147,153,159,160,168,169) At the molecular level, the presence of functional tetracycline resistance genes (*tet45*, *tetB*) was identified through WGS, PCR and on isolated plasmids. (114,116,169) The genes *erm(A*) and *erm(B)* mediating macrolide resistance were also detected in *Bacillus cereus sensu lato* isolates. (116,170)

Despite an increasing number of clinically relevant antimicrobial resistances, broad knowledge about antimicrobial susceptibility of *Bacillus cereus sensu lato* is still limited. Although the Clinical and Laboratory Standards Institute (CLSI) proposed breakpoints for *Bacillus* spp. in 2007, (171) the European Committee on Antimicrobial Susceptibility Testing (EUCAST) did not introduce its own breakpoints and zone diameters until 2021. (172) Consequently, the susceptibility testing itself of *Bacillus cereus sensu lato* is still scarcely investigated.

Hypotheses and aims of the dissertation project

Bacillus cereus sensu lato is a ubiquitous species that is present in the food chain from farm to fork, it can be found in our everyday materials, including food packaging, and it causes food poisoning but also serious non-gastrointestinal infections, which typically require precise anti-infective therapy. The global spread of antimicrobial resistance represents a significant challenge to global health. Consequently, surveillance of antimicrobial susceptibility should not be limited to highly virulent pathogens (e.g., the ESKAPE bacteria), but should also encompass neglected pathogens such as *Bacillus cereus sensu lato*. The increasing number of studies

reporting antimicrobial resistances in *Bacillus cereus* strains contributes to completing this gap. However, due to its reputation as a foodborne pathogen, most studies are limited to food samples and data on clinical isolates, especially isolates from non-gastrointestinal infections, is scarce worldwide. Therefore, a total of 93 patient isolates, collected over several years at the Department of Bacteriology at the Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine in Graz, Austria, were analyzed for their phylogenetic affiliation and antimicrobial susceptibility. Furthermore, 138 isolates were obtained from 48 patients participating in a clinical trial investigating the contamination of cranial orthoses. Additionally, isolates were also obtained from the respective hospital environment. The healthcare sector and hospitals are hotspots for the development and spread of antibiotic-resistant bacteria. The comparison with environmental isolates is therefore a valid approach to monitor and evaluate i) the origin of antibiotic-resistant bacteria, ii) the clinical significance of these bacteria, iii) and the future dissemination beyond the healthcare sector. In the food sector, *Bacillus cereus sensu lato* has been the subject of continuous assessment, given the potential for antibiotic-resistant bacteria to originate from livestock breeding and manure slurry. Hence, it may be preferable to select samples that are less susceptible to contamination from livestock breeding. Food packaging materials and the associated industrial processes provide a suitable reference condition as an environmental source of *Bacillus cereus sensu lato*, which is then in close contact with humans via the food supply chain. A total of 26 isolates from industrial biofilms and process water from an Austrian packaging material manufacturer, as well as 80 isolates from food packaging material, were selected for comparison with isolates from patients in terms of phylogenetic affiliation and antimicrobial susceptibility. The corresponding packaging material isolates have already been described in Schmid et al. (138) and in “Phylogenetic, Phenotypical and Toxigenic Characterization of Bacillus cereus Isolates from Packaging Materials” (master’s thesis 2020). However, the present study includes updated susceptibility testing.

The findings provide evidence regarding the prevalence of antimicrobial resistance in the *Bacillus cereus* group in both patients and industrial/food-associated environmental samples from southern Austria. Moreover, the analysis of distinct phylogenetic traits in patients, hospital environments, and industrial samples provides a more accurate representation of the significance of this opportunistic pathogen in the respective environment. The project encompassed a broad spectrum of *Bacillus cereus sensu lato* strains, but no particular attention was paid to *Bacillus anthracis* or *Bacillus mosaicus* subsp. *anthracis* or the biovar Anthracis.

This decision was made due to the rarity of the anthrax-causing strains, the low incidence of reported cases in general, and the necessity of rigorous biosafety measures. (173)

A major focus of this dissertation has become the evaluation of antimicrobial susceptibility testing of *Bacillus cereus sensu lato* in general. Since standardized breakpoints and zone diameters from EUCAST have only recently become available, there is limited knowledge on phenotypes in the *Bacillus cereus* group that could interfere with accurate susceptibility testing. In the course of this project, a prima facie vancomycin-resistant phenotype was identified. Routine methods used in AST resulted in a phenotype highly resistant to vancomycin. To date, vancomycin resistance in *Bacillus cereus sensu lato* has hardly been investigated. Although some studies reported resistant isolates, explanations for their observations were not provided. (48,174) In light of the putative vancomycin resistance, a significant emphasis was placed on the validation, characterization, and molecular background of the phenotype, leading to the description of a motility mechanism rather than a true resistance mechanism. As a representative of the pseudo-resistant phenotype, the food-packaging isolate BC70, which appeared to be highly resistant, was selected for microscopic and gene expression analysis. The elucidation of this unique mechanism during susceptibility testing ultimately came at the expense of the molecular detection of other resistance-mediating genes in *Bacillus cereus sensu lato*.

Materials and Methods

Samples and isolation of *Bacillus cereus sensu lato* strains

This dissertation project encompassed *Bacillus cereus sensu lato* isolates from patient samples from microbiological diagnostics, patients participating in a clinical trial regarding cranial orthoses, the respective hospital environment, food packaging materials and industrial environments (biofilms and process water).

All isolated strains of *Bacillus cereus sensu lato* were stored at - 80 °C using the Microbank system (VWR International GmbH, Vienna, Austria) and the Cryobank system (Serobac GmbH, Vienna, Austria). For most experiments, the *Bacillus cereus* type strain ATCC 14579 was used as a reference.

Patient isolates

Isolates from routine patient samples were collected by the Department of Bacteriology at the Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine at the Medical University of Graz, Austria, as part of the microbiological routine diagnostics of patient samples. The patient samples came from the University Hospital Graz, different outpatient clinics and physicians in private practice from the region Styria. The samples were obtained from 2020 to 2024 and included wound swabs and various other swabs, urine, aspirates, tissue, sputum, positive blood cultures, feces and other undefined samples. The isolation of *Bacillus cereus sensu lato* was carried out with routine methods in the diagnostic laboratory depending on the type of sample. The isolates were subsequently provided for further analysis. In general, isolates were collected regardless of their relevance to the disease and putative sample contaminations were also included. Nevertheless, many of the isolates were associated with the patients' infectious diseases, such as isolates from positive blood cultures. All isolates were blinded prior to downstream analyses, and no related patient information was obtained from the samples. Accordingly, ethical approval was not obtained for the isolates. A total of 87 isolates from various patient samples were collected. The 87 isolates from various samples and the corresponding microbiological patient reports were retrospectively evaluated for the information present on the microbiological report. In addition, six strains previously isolated from positive blood cultures and part of the institute's strain collection were included in the study. Finally, 93

isolates from routine patient samples were investigated, including a total of eight isolates from positive blood cultures. All personal data was anonymized and analysis only focused on the reporting practice of *Bacillus cereus sensu lato* and corresponding AST.

In addition to patient isolates from routine microbiological diagnostics, *Bacillus cereus sensu lato* isolates were also collected from patients during a prospective study regarding cranial orthoses for infants. The study was conducted by the Division of Oral and Maxillofacial Surgery, which is part of the Department of Dental Medicine and Oral Health at the Medical University of Graz, Austria. The ethical approval was obtained by the principal investigators. The study was intended to investigate the microbial colonization of the scalp and the helmet during helmet molding therapy of infant patients with unilateral (plagiocephaly) or bilateral (brachycephaly) flattening of the occiput. Female, male or non-binary patients were included. The following major exclusion criteria were applied: known skin conditions (e.g., psoriasis, atopic eczema, etc.), an age exceeding 12 months at the commencement of therapy, and a duration of therapy below 4 months. Bacterial isolates were sampled with swabs (eSwab, Copan Italia S.p.A., Brescia, Italy) of the scalp at the start of helmet molding therapy, with swabs of the cranial orthosis (helmet) at each check-up visit at the outpatient clinic of the Division of Oral and Maxillofacial Surgery, and with a final swab of the scalp six months after the end of therapy, if applicable. The swabs were taken from patients by the investigators at the Division of Oral and Maxillofacial Surgery and subsequently delivered to the Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine. In the microbiological laboratory, the swabs were streaked on Columbia Agar with 5% Sheep Blood (Col-S, Becton Dickinson Austria GmbH, Vienna, Austria) followed by incubation at 37 °C overnight. Putative colonies of *Bacillus cereus sensu lato* were then isolated and confirmed with MALDI-TOF MS using the VITEK MS platform (bioMérieux Austria GmbH, Vienna, Austria). A total of 138 isolates of *Bacillus cereus sensu lato* were collected from 48 patients. As the outcomes of the clinical trial are not included in this dissertation, all isolates from all patients, including dropouts, were included in the dissertation project.

Hospital isolates – outpatient clinic

The study on cranial orthoses also included microbiological sampling of the outpatient clinic environment via swabs (eSwab, Copan Italia S.p.A.), Count-Tact® agar (bioMérieux Austria

GmbH) and air sampling with the MAS 100 NT (MBV AG, Staefa, Switzerland) using tryptic soy agar plates (VWR International GmbH). A total of 16 isolates of *Bacillus cereus sensu lato* were collected from a variety of surfaces, including clothing, the interior of a fume hood, and the corresponding rubber sealing. Additionally, isolates were obtained from a grinding machine, air sampling, and 70% ethanol for disinfection purposes. The swabs were streaked on Col-S agar (Becton Dickinson Austria GmbH) and all agar plates were incubated at 37 °C overnight. Putative colonies of *Bacillus cereus sensu lato* were then isolated and confirmed with MALDI-TOF MS.

Industrial isolates (food packaging, biofilms, process water)

The industrial isolates comprised strains from food packaging samples and from the respective industrial environment of the manufacturing site. The food packaging samples were composed of fiber-based materials (e.g. cardboard, paper) and were provided by four distinct manufacturing facilities in Europe. All samples came from the food packaging sector reflecting primary and secondary food packaging. The samples were taken under sterile conditions by instructed workers and wrapped in sterile aluminum foil to avoid contamination. Afterwards the samples were sent to the microbiological laboratory in sealed plastic bags. One gram of each sample was weighed and evenly homogenized with 99 ml of sterile buffered peptone water (Oxoid, Thermo Fisher Diagnostics Austria GmbH, Vienna, Austria) in sterile polyethylene bags (VWR International GmbH) using a BagMixer 400 SW (Interscience, Wiesbaden, Germany). Afterwards, 0.5 ml of the fiber suspension were transferred on Brilliance Bacillus cereus agar (Oxoid, Thermo Fisher Diagnostics Austria GmbH). The plates were incubated at 37 °C for 48 h. Homogenized packaging materials in buffered peptone water were enriched for *Bacillus cereus sensu lato* at 37 °C for 24 h. Then, the samples were streaked again on Brilliance Bacillus cereus agar to detect *Bacillus cereus sensu lato*. A total of 80 isolates were isolated from 41 different food packaging samples and confirmed as *Bacillus cereus sensu lato* with MALDI-TOF MS. (138,175)

Samples of biofilm and process water were collected at a manufacturing site for fiber-based food packaging materials in southern Austria between 2018 and 2020. Biofilm was collected in 50 mL CELLSTAR® Polypropylen Tubes (Greiner Bio-One International, Kremsmünster, Austria) and process water was collected in 250 mL Corning® Gosselin™ Water Sampling Octagonal

Polyethylene terephthalate (PET) bottles, containing 120 mg/L sodium thiosulfate (VWR International GmbH). The samples were taken by instructed workers at the manufacturing site and were subsequently brought to the microbiological laboratory and kept at 4 °C. Biofilm and process water were cultured on Col-S agar (Becton Dickinson Austria GmbH) and putative colonies of *Bacillus cereus sensu lato* were isolated and confirmed with MALDI-TOF MS (bioMérieux Austria GmbH). A total of 26 strains of *Bacillus cereus sensu lato* were isolated. Of these, 22 originated from seven samples of industrial biofilms, while the remaining four originated from one process water sample.

Assignment to phylogenetic *panC* groups

The stored isolates were streaked on Col-S agar (Becton Dickinson Austria GmbH) and incubated at 30 °C overnight. On the following day, 5 mL of tryptic soy broth (Oxoid, Thermo Fisher Diagnostics Austria GmbH) were inoculated with a single colony and incubated again at 30 °C overnight. For DNA extraction, 400 µL of the bacterial culture were pelleted at 3000 g for 10 minutes. The pellet was resuspended in 200 µL of autoclaved TE buffer (30mM Tris-HCl, 1mM EDTA, pH8) and the DNA was extracted using the innuPREP Bacteria DNA Kit (IST Innuscreen GmbH, Berlin, Germany). The DNA was eluted in 50 µL of elution buffer. Successful extraction was verified by measuring the amount of extracted DNA using the Nanodrop2000 (Thermo Fisher Scientific, Vienna, Austria). The DNA was stored in elution buffer at -20 °C until needed. (138)

The partial *panC* gene was amplified using the following primer sequences for groups I to VI according to Guinebretière et al. (2): 5′-TYGGTTTTGTYCCAACRATGG-3′ (forward degenerated primer) and 5′-CATAATCTACAGTGCCTTTCG-3′ (reverse primer). Initially, all isolates were tested for the phylogenetic groups I to VI. If PCR amplification was unsuccessful, the isolate was tested for group VII using the alternative reverse primer 5′-CATAATCAACTATACCGTTTG-3′. The PCR was carried out in a Biometra Trio 48 thermocycler (Analytik Jena GmbH+Co. KG, Jena, Germany) in a final volume of 25 µl containing 1X NEB Q5 Reaction Buffer (New England Biolabs GmbH, Frankfurt am Main, Germany), 200 µM of each dNTP (dNTP Mix 10mM, Biozym Biotech Trading GmbH, Vienna, Austria), 0.5 µM of each primer, 0.5 IU of NEB Q5 High Fidelity DNA Polymerase (New England Biolabs GmbH) and 1 µl DNA template. The temperature protocol included an initial denaturation at 98 °C for 30 s followed by 30 cycles of 98 °C for 10 s,

61 °C for 30 s, 72◦C for 30 s and a final extension at 72 °C for 2 min. The successful amplification was verified by agarose gel electrophoresis using a 1.6% agarose (w/v) gel. For agarose gel preparation, 1X TAE buffer (50X stock solution TAE buffer, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA), LE agarose (Biozym Biotech Trading GmbH) and Midori Green Advance (NIPPON Genetics Europe GmbH, Düren, Germany) were used. DNA was applied using the gel loading dye, purple (6X), no SDS (New England Biolabs GmbH). The 100 bp DNA ladder (New England Biolabs GmbH) was used as a size marker. Electrophoresis was performed with 80V for 45 minutes and images were taken with the GelDoc™ Go Imaging System (Bio-Rad Laboratories GmbH, Vienna, Austria). Upon successful amplification, the MSB Spin PCRapace Kit (Invitek Diagnostics Germany, Berlin, Germany) was used for DNA purification. Purified PCR products were sent to Eurofins Genomics Germany GmbH (Cologne, Germany) for Sanger sequencing using the sequencing primer 5′-ATAATCTACAGTGCCTTTCG-3′. In the case of an isolate belonging to group VII, the alternative sequencing primer 5′-ATAATCAACTATACCGTTTG-3′ was used. (138)

Sequence data analysis for the assignment to groups I to VII was done by uploading FASTA sequence data to an online homology search algorithm (https://toolcereusid.shinyapps.io/Bcereus/). (2)

Additionally, the obtained sequences of the *panC* gene were analyzed with the Btyper3 software tool. It is a command line tool, which includes the adjusted eight-group framework of *panC* typing and the most recent nomenclature of *Bacillus cereus sensu lato* based on the proposals of Carroll et al. (43,44). The use of Btyper3 is more labor-intensive due to the restriction of analyzing DNA sequences in FASTA format one by one. Consequently, only isolates belonging to phylogenetic groups II, III, V, and VI, as determined with the seven-group framework, underwent further analysis with Btyper3 to confirm the phylogenetic group. Isolates of these groups showed overlapping assignments when comparing the seven-group and the eight-group framework (Table 1). For the other phylogenetic groups, results of both *panC* typing tools were shown to be consistent. (44)

Antimicrobial Susceptibility Testing

Disk diffusion testing

The antimicrobial susceptibility was evaluated using the disk diffusion method in accordance with current EUCAST guidelines. (176) For this purpose, single colonies of fresh overnight cultures on Col-S agar (Becton Dickinson Austria GmbH) were picked with a sterile cotton swab. A bacterial suspension equal to McFarland standard of 0.5 was prepared in 0.9% sterile saline solution (w/v). The correct turbidity of the inoculum was verified with the DensiCHEKTM Plus densitometer (bioMérieux Austria GmbH). Sterile swabs were dipped into the suspension to evenly inoculate Mueller Hinton E agar plates (bioMérieux Austria GmbH). Afterwards, paper disks containing the following antimicrobial agents were applied (BBL™ Sensi-DiscTM, Becton Dickinson Austria GmbH): vancomycin (5 µg), clindamycin (2 µg), erythromycin (15 µg), linezolid (10 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg). The plates were incubated at 36 ±1 °C for 16 to 20 h. After incubation, the diameter of the inhibition zone was determined. As there are no comments on the interpretation of distinct phenotypes in antimicrobial susceptibility of *Bacillus cereus sensu lato,* zone diameters were reported as measured. For interpretation of zone diameters, the current version of the EUCAST clinical breakpoint tables was used. (177) As a routine quality control, the following bacterial strains were used as recommended by EUCAST and evaluated in accordance with the relevant standards (178): *Staphylococcus aureus* ATCC 29213 (clindamycin, erythromycin, linezolid, ciprofloxacin, levofloxacin), *Escherichia coli* ATCC 25922 (imipenem, meropenem), *Enterococcus faecalis* ATCC 29212 (vancomycin). (179) Isolates were classified as wild type if no resistance to any of the tested antimicrobial agents was observed.

Gradient test method (Etest®) for vancomycin

The gradient test method was performed to determine the minimum inhibitory concentration (MIC) of isolates exhibiting a phenotype resistant to vancomycin. Similar to the disk diffusion method, Mueller Hinton E agar plates (bioMérieux Austria GmbH) were inoculated with a bacterial suspension equal to a McFarland standard of 0.5. Gradient tests containing increasing concentrations of vancomycin were applied (Etest® Vancomycin, bioMérieux Austria GmbH; MIC Test strip Teicoplanin, Liofilchem, Roseto degli Abruzzi, Italy). The plates were incubated at 36 ±1 °C for 16 to 20 h. The MIC was determined as described in the manufacturer's manual and

susceptibility was interpreted according to the current version of the EUCAST clinical breakpoint tables. (177) In general, the MIC is defined by the intersection of the pointed end of the ellipse (inhibition zone) and the Etest® strip. If the inhibition zone intersected the strip between two values, the higher concentration was noted. As for the disk diffusion, there are no comments on the interpretation of distinct phenotypes in antimicrobial susceptibility of *Bacillus cereus sensu lato.* Therefore, MICs were reported as measured.

Broth microdilution for vancomycin

As reference methods for AST, broth microdilution and agar dilution were used to confirm putative vancomycin resistance. Broth microdilution was performed according to EUCAST recommendations. (180) In brief, Mueller Hinton broth (Carl Roth GmbH, Karlsruhe, Germany) was supplemented with vancomycin hydrochloride (Heraeus Medical GmbH, Wehrheim, Germany) to reach the following final concentrations: 0.125 mg/L, 0.25 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L and 16 mg/L. Bacteria were pre-cultured on Col-S (Becton Dickinson Austria GmbH) agar at 37 °C overnight and a bacterial inoculum equal to McFarland standard of 0.5 was prepared in 0.9% sterile saline solution (w/v). In microtiter plates, 200 µL Mueller Hinton broth were inoculated to a final concentration of $5x10^5$ CFU/mL. The growth was monitored at 37 °C for 24 h using the Bioscreen C system (Oy Growth Curves Ab Ltd., Turku, Finland) and visually evaluated at the end. Quality control of the correct inoculum was performed as recommended by EUCAST. All concentrations were tested in three to five replicates and growth controls without vancomycin were included.

Agar dilution for vancomycin

Agar dilution assays were performed according to the recommendations of EUCAST. (181) Vancomycin hydrochloride (Heraeus Medical GmbH) was added to melted Mueller Hinton agar (Carl Roth GmbH) for the following final concentrations: 0.125 mg/L, 0.25 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L and 16 mg/L. Afterwards, agar plates were poured. Bacteria were pre-cultured on Col-S (Becton Dickinson Austria GmbH) at 37 °C overnight. An inoculum of 10^4 colony-forming units (CFU) per spot was spotted onto Mueller Hinton agar (MH) plates containing vancomycin using a micropipette. The inoculum was prepared in a 0.9% sterile saline

solution. Plates were incubated at 36 ±1 °C for 18 h. Growth was visually evaluated. The MIC was the lowest concentration of the antimicrobial agent that completely inhibited visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot. (181) All concentrations were tested in 5 replicates and growth controls without vancomycin were included.

Microscopic Imaging

Time-lapse microscopy

The growth behavior of the food packaging isolate BC70, which exhibited a highly resistant phenotype to vancomycin in agar diffusion assays, was visualized during agar diffusion susceptibility testing with time lapse microscopy. For this purpose, the gradient test method for vancomycin was used, as a larger inhibition zone could be observed. Time-lapse microscopy was performed overnight, using the Nikon High Content Screening System (Nikon Eclipse Ti2 microscope, Nikon Gmbh, Vienna, Austria) with microscope cage incubator (Okolab, Nikon Gmbh). Vancomycin gradient tests (Etest® Vancomycin, bioMérieux Austria GmbH) were applied on inoculated Mueller Hinton E agar plates (bioMérieux Austria GmbH) following the EUCAST recommendations. (176) Then, the plates were incubated at 37 °C for 24 h in the cage incubator for live-cell imaging. Images of the region of interest were automatically taken every 30 min with Plan Apo 4x objective lenses. Images were taken with Zyla sCMOS camera (Andor, Oxford Instruments GmbH, Wiesbaden, Germany) and image processing was carried out with NIS elements viewer and Fiji (v2.15.0). (179)

Phase-contrast microscopy

In addition to time-lapse microscopy, the morphology of the cells at the inner rim of the inhibition zone was investigated using phase-contrast microscopy. Initially, the BC70 isolate was grown on Mueller Hinton E agar (bioMérieux Austria GmbH) plates with a vancomycin gradient test (Etest® Vancomycin, bioMérieux Austria) overnight following EUCAST recommendations. (176) Subsequently, an area of approximately 1.5 by 1.5 cm of the designated region of interest on the plates was excised from the agar plate using a scalpel. This region of interest contained the rim of the inhibition zone of the vancomycin gradient test. The upper 2 to 3 mm of the cutout agar

were prepared by cutting off the excess agar at the bottom, placed on a microscope slide and covered with a cover slip. The vancomycin inhibition zone of the reference strain ATCC 14579 was prepared following the same protocol. Additionally, the morphology of cells from the bacterial lawn, where no inhibition by vancomycin took place, was evaluated. Given the high cell density in the area, direct microscopy was not a feasible option. Instead, the cells were collected with a sterile cotton swab and immersed in a drop of 0.9% sterile saline solution on the microscopic slide and covered with a cover slip. Phase contrast microscopy was carried out on the Axio Lab.A1 microscope (Carl Zeiss GmbH, Vienna, Austria) using 40x phase contrast objective lenses. (179)

Testing for swarming motility

To test possible swarming motility of the BC70 isolate during vancomycin susceptibility testing, swarming cells of the strain were stained with Leifson flagella stain. For the Leifson dye solution, three separate stock solutions were prepared and mixed in equal parts. The first solution was prepared by dissolving 1.5 g NaCl (Carl Roth GmbH) in 100 mL deionized water. For the second solution, 3.0 g tannic acid (Carl Roth GmbH) were dissolved in 100 mL deionized water. The third solution contained 1.0 g basic fuchsine (Carl Roth GmbH) in 100 mL of 96% ethanol. The final dye solution was kept at 4 °C in the refrigerator and was stable for up to two months. (182) The staining protocol was established with *Proteus mirabilis* DSM 788, which exhibits a strong swarming motility even on common semisoft agar plates with approximately 1.5% agar. The glass sides were pre-cleaned with 96% ethanol and lint free wipes. Residual ethanol was removed by heating the glass slide with a Bunsen burner. Then cells of BC70 from the inhibition zone edges of the vancomycin gradient test plates (37 °C, overnight) were picked up with a loop and carefully immersed in 5.0 µL of deionized water on a glass slide to preserve the flagella. The suspension was spread on the glass slide only by tilting the slide, as the flagella are easily destroyed, and then dried at room temperature. A rectangle was drawn with a wax pencil around the dried sample and covered with Leifson dye solution for 15-20 minutes. The slides were subsequently washed with tap water and cells were counterstained with Loeffler's methylene blue solution (Merck Chemicals and Life Science GmbH, Vienna, Austria) for 2 min. The stained slides were covered with a cover slip and subsequently examined under the microscope. Bright-

field microscopy was carried out on the Axio Lab.A1 microscope (Carl Zeiss GmbH) using 100x objective lenses and oil immersion. (179)

As a positive control for the swarming phenotype, the BC70 isolate and the ATCC 14579 strain were grown in tryptone broth containing 1% (w/v) tryptone (Oxoid, Thermo Fisher Diagnostics Austria GmbH) and 0.5% (w/v) NaCl (Carl Roth GmbH) overnight. Then, 5.0 µL of the liquid cultures were spotted on tryptone swarming agar containing 0.7% (w/v) agar-agar Kobe I (Carl Roth Gmbh) and incubated at 37 °C in a humidified chamber until swarming motility phenotypes were observed (48 to 96h). (183) Afterwards, swarming cells were collected with a sterile cotton swab and immersed in tryptone broth, thereby obtaining a bacterial suspension with an optical density equal to that of the McFarland standard of 0.5. Swarming bacteria were then sub-cultured by spotting 5.0 µL on tryptone swarming agar and Mueller Hinton swarming agar (MHS) to obtain stable swarming populations of the BC70 isolate and the reference strain ATCC 14579. Each spot contained approximately 1 x 10^6 CFU. Mueller Hinton swarming agar plates were prepared by adding 0.7% (w/v) agar-agar Kobe I (Carl Roth GmbH) to Mueller Hinton broth (Carl Roth GmbH). (179)

Colony expansion assay

The colony motility of BC70 on different growth media was evaluated in comparison to the reference strain ATCC 14579. Bacterial strains were grown on Col-S agar (Becton Dickinson Austria GmbH) overnight at 37 °C. The following day, a bacterial suspension equivalent to McFarland 0.5 was prepared in 0.9% sterile saline solution and 5.0 µL were spotted on Col-S agar (Becton Dickinson Austria GmbH), Mueller Hinton E agar (MH, bioMérieux Austria GmbH) and TrA swarming medium containing 1% (w/v) tryptone (Oxoid, Thermo Fisher Diagnostics Austria GmbH), 0.5% (w/v) NaCl (Carl Roth GmbH) and 0.7% (w/v) agar-agar, Kobe I (Carl Roth GmbH). All plates were incubated at 37 °C and evaluated every 24 h. The TrA plates were incubated in a humidified chamber. All measurements were done in triplicates. (179) In addition, BC70 cells were also pre-cultured on TrA plates to obtain a stable swarming population prior to inoculation and then spotted onto TrA plates as previously described.

Gene expression analysis

Gene expression analysis was used to elucidate the underlying molecular mechanisms that may contribute to the putative vancomycin resistance in certain strains of *Bacillus cereus sensu lato*. The food packaging isolate BC70 during vancomycin gradient test exposure was selected for comparative analysis with the swarming phenotype of BC70, ordinary growth on Mueller Hinton E agar and the reference strain ATCC 14579, which was also grown with vancomycin gradient test exposure.

RNA isolation

The BC70 isolate and ATCC 14579 were cultured on Mueller Hinton E agar plates (bioMérieux Austria GmbH) with vancomycin gradient tests (Etest®, bioMérieux Austria GmbH) following the EUCAST guidelines. Furthermore, a swarming phenotype of the BC70 isolate was prepared on Mueller Hinton swarming agar (MHS) and cultures of BC70 were grown on normal Mueller Hinton E agar plates (MH, bioMérieux Austria GmbH) without vancomycin. For each condition, two to four biological replicates were pooled. For vancomycin gradient test plates, cells were taken from both sides of the Etest® strip at the innermost inhibition zone edges (Figure 1). Bacterial cells from a total of four plates with a vancomycin Etest® strip were pooled. The swarming cells were picked from the outermost edges of swarming colonies from two different agar plates. The swarming phenotype was confirmed by staining the flagella immediately after sampling as described before. A total of 5 to 6 colonies were taken from each of the two normal Mueller Hinton E plates. All samples were taken after exactly 13 h of incubation, as active motility on vancomycin gradient test plates towards the inhibition zone could be observed by time-lapse microscopy at this time. RNA extraction was performed in technical duplicates. For each replicate, around 1.8 OD_{600} of cells were taken with sterile loops and transferred into a 2 mL tube containing 800 µL RNAprotect Bacteria Reagent (QIAGEN GmbH, Hilden, Germany) and 400 µL RNase-free phosphate buffered saline (Invitrogen™, Thermo Fisher Diagnostics Austria GmbH). Afterwards, the cells were pelleted at 5000 g for 10 min and the supernatant was discarded. The pellet was resuspended with 100 µL lysozyme (15 mg/mL, Carl Roth GmbH) and 15 µL proteinase K (QIAGEN GmbH) and incubated for 3h at room temperature prior to cell lysis. First, the RNeasy Lysis Buffer (QIAGEN GmbH) was supplemented with 1% β-mercaptoethanol (Carl Roth GmbH). Then the cells were mixed with 700 µL of lysis buffer

followed by mechanical disruption for 6 x 30s at 4260 rpm (maximum speed) in a BeadBlaster (Biozym Biotech Trading GmbH) with cooling on ice in between. After quick centrifugation, the resulting supernatant was collected and mixed with the same amount of 80% ethanol. RNA was further purified with the RNeasy Mini Kit (QIAGEN GmbH) according to the manufacturer's protocol. (179)

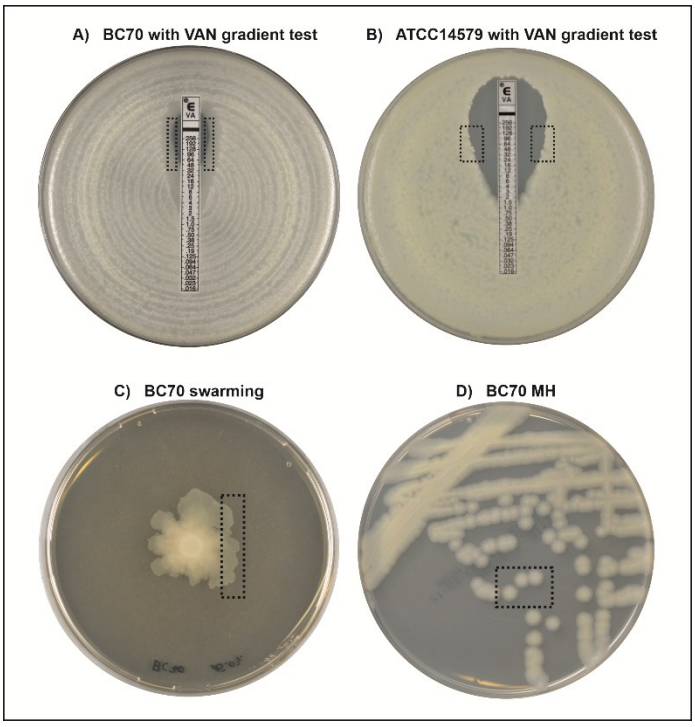

Figure 1: Sampling sites for RNA extraction prior to RNA-Seq analysis. All conditions were harvested after exactly 13 h of incubation. Sampling areas are indicated with a dotted line. A) putatively resistant isolate BC70 with vancomycin (VAN) gradient test. Bacterial cells were collected from the more translucent area at the innermost edges of the inhibition zone parallel to the gradient strip. B) Reference strain ATCC 14579 with vancomycin gradient test served as control. As for BC70, cells were collected from the innermost edges of the inhibition zone. C) BC70 on Mueller Hinton swarming agar (BC70 swarming). Swarming cells were collected from the migrating edge of the swarming colony. D) Normal colonies of BC70 on Mueller Hinton agar (BC70 MH). [Reproduced and adapted from Schmid et al. (179).]

Library Prep and Oxford Nanopore Sequencing

Isolated RNA was quantified using the Qubit fluorometer (Thermo Fisher Diagnostics Austria GmbH) and technical replicates of each condition were pooled afterwards to obtain sufficiently high amounts of RNA. Pooled samples were then further purified with the TURBO DNA-free kit (Thermo Fisher Diagnostics Austria GmbH) and enriched with RNAClean XP beads (Beckman Coulter GmbH, Vienna, Austria). rRNA depletion was carried out with the NEBNext rRNA Depletion Kit for bacteria (New England Biolabs GmbH). Subsequent polyadenylation was performed according to the Oxford Nanopore protocol for polyadenylation with *E. coli* poly(A) polymerase (New England Biolabs GmbH). The direct cDNA Kit (Oxford Nanopore) and the Native Barcoding Expansion 1-12 (Oxford Nanopore Technologies, Oxford, United Kingdom) were used for library prep. In general, Qubit measurements were reduced to a minimum to maintain sufficiently high amounts of RNA or cDNA for sequencing. Transcriptome sequencing was done with two technical replicates on the MinION Mk1B equipped with the FLO-MIN106 flow cell (Oxford Nanopore Technologies) and MinKnow 22.12.7 software. The minimal read length was set to 200 bp and a minimum quality score value of 7. Between technical replicates, the flow cell was washed according to the Oxford Nanopore protocol. (179)

Data Analysis

Sequencing data was basecalled using Guppy 6.4.6 in super-accurate mode. Passed reads from replicates were analyzed using EPI2ME Desktop (v5.0.2) with the nextflow transcriptomes workflow (v0.2.0). The NCBI RefSeq assembly reference genome and the RefSeq annotation of *Bacillus cereus* (genome assembly ASM222028v1, RefSeq GCF_002220285.1, strain FORC47) were used for reference-based transcriptome assembly and reference annotation, respectively. All analyses were run with default parameters. For differential expression analysis, data from the respective conditions were compared with each other, e.g. BC70 isolate against ATCC 14579, each on vancomycin gradient test plates. The gene IDs of significantly differentially expressed genes (false discovery rate < 0.05 and fold change ≥ ±2) were matched with gene names using DAVID. (184) Functional analysis for gene ontology (GO) and cluster of orthologous groups (COG) analysis were performed using FUNAGE-Pro. (185) Furthermore, genes were manually classified according to their proposed function using the KEGG database, analogous genes in

other *Bacillus cereus* group strains and profound literature research. Gene expression data was deposited in the NCBI Gene Expression Omnibus repository (accession no. GSE253142). (179)

Statistical analysis of AST

Data on antimicrobial susceptibility in different populations was statistically analyzed in GraphPad Prism (v9.5.1, GraphPad Software, Boston, MA, USA). For comparing the frequencies of resistant phenotypes, the Pearson's chi-squared test of independence (chi-square test) with a confidence interval of 95% was used. Chi-square calculations were only valid when all expected values were greater than 1.0 and at least 20% of the expected values were greater than 5. To compare the resistances to individual antimicrobial agents, a Fisher’s exact test with a confidence interval of 95% and a two-sided P value was employed.

Data visualization

Data visualization was carried out in GraphPad Prism (v9.5.1, GraphPad Software) and CorelDraw Graphics Suite 2019 (Corel Corporation, Ottawa, Canada).

Results

In the Results section, the phylogenetic classification and the reported antimicrobial resistances of the *Bacillus cereus sensu lato isolates* are presented separately for each sample set. The subsequent parts address the phenotypic and molecular characterization of a sliding motility phenotype that posed a challenge to accurate vancomycin susceptibility testing.

Patient isolates – samples from microbiological routine diagnostics

All 93 isolates from different patient samples could be assigned to their respective phylogenetic groups. Based on the sequence of the *panC* gene, five different phylogenetic groups were identified, with group III being the most abundant (Figure 2). About 38% of the isolates (35 isolates) belonged to group III, which comprises the genomospecies of *Bacillus mosaicus* with the established lineages of subsp. *anthracis* and subsp. *cereus*. Group III is followed by groups IV, II and V, which account for 28% (26 isolates), 23% (21) and 11% (10), respectively. A single isolate (1.08%) was assigned to group VI, comprising *Bacillus mycoides* and *Bacillus paramycoides* according to the genomospecies framework. In general, the phylogenetic affiliation of the strains appears to exhibit a relatively even distribution, with no phylogenetic group clearly dominating. Eight isolates were obtained from positive blood cultures, and no pattern was observed with regard to these isolates. Specifically, three isolates were identified as belonging to group II, two isolates each were classified as belonging to groups III and IV, and one isolate was assigned to group V.

Upon confirming those groups of the seven-group scheme that overlap in the eight-group framework (II, III, V, and VI), the Btyper3 software tool identified a single misclassification of an isolate. The respective isolate of group III was re-classified as group II using the adjusted eight-group framework.

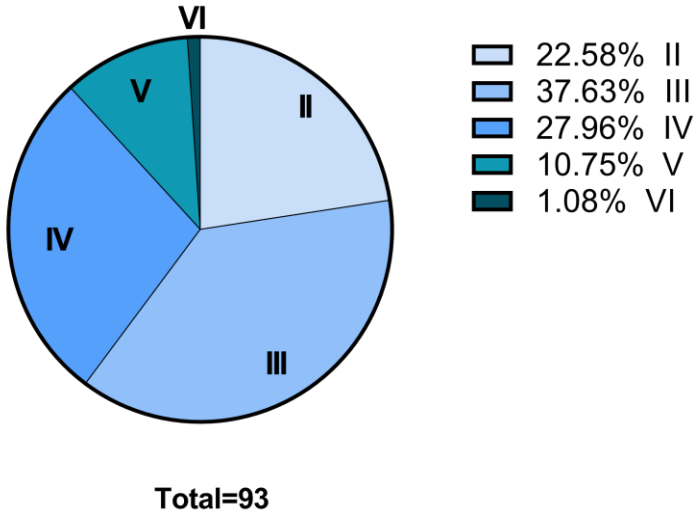

Figure 2: Phylogenetic distribution of *Bacillus cereus sensu lato* from patient samples. The isolates were obtained from microbiological routine diagnostics of patient samples, regardless of their relevance to the disease and including putative sample contamination. Phylogenetic assignment of the isolates (n = 93) was based on the *panC* gene sequence. Only the eight-group adjusted *panC* scheme is presented. (2,44)

The antimicrobial susceptibility testing using disk diffusion revealed several resistance phenotypes to clinically relevant antibiotics in the patient isolates (Table 2). The most prevalent resistance was observed for erythromycin, with 31 isolates (33.33%) exhibiting resistance in disk diffusion testing. For one of the isolates, no inhibition was discernible around the erythromycin disk. Regarding the carbapenems, 22 (23.66%) and 16 (17.20%) isolates were reported resistant to imipenem and meropenem, respectively. For both antimicrobial agents, small zone diameters and the documented resistance frequently resulted from single colonies, which grew within the inhibition zone. Furthermore, 10 out 93 isolates (10.75%) were reported to be resistant to vancomycin, with zone diameters ranging from 7 to 9 mm, which is below the breakpoint of 10 mm. The data indicated very low prevalence of resistance to clindamycin, levofloxacin, ciprofloxacin, and linezolid, with only three, two, one, and one of the isolates exhibiting resistance, respectively. For ciprofloxacin and levofloxacin, the resistant isolates exhibited zone diameters clearly below the breakpoint (23 mm), with values ranging from 14 to 16 mm. All non-

resistant isolates (92 and 91, respectively) were indicated as “susceptible, increased exposure” to ciprofloxacin and levofloxacin in accordance with the EUCAST clinical breakpoints.

Table 2: Antimicrobial susceptibility of 93 isolates of *Bacillus cereus sensu lato* from patient samples. The isolates were obtained through microbiological diagnostics of routine samples from patients. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints.

	susceptible	**susceptible, increased exposure**	**resistant**
clindamycin	90	-	3 (3.23%)
erythromycin	62	-	31 (33.33%)
ciprofloxacin	0	92	1 (1.08%)
levofloxacin	0	91	2 (2.15%)
vancomycin	83	-	10 (10.75%)
linezolid	92	-	1 (1.08%)
imipenem	71	-	22 (23.66%)
meropenem	77	-	16 (17.20%)

Of the 93 isolates derived from patient samples, 41 (44.09%) were classified as wild type, exhibiting no resistance to any of the tested antimicrobial agents (Figure 3). Resistance to only one of the antimicrobials was observed in 31 isolates, representing one-third of the total number of isolates tested. Ten of the isolates demonstrated resistant phenotypes to two antimicrobial agents (10.75%), while 11 isolates exhibited resistance to three or more antimicrobial agents (11.83%). Specifically, two isolates were reported to be resistant to four antimicrobials: erythromycin, vancomycin, imipenem, and meropenem. All isolates with resistance to three or more antimicrobial agents were resistant to erythromycin. Among the isolates that were resistant to erythromycin, only one isolate exhibited concomitant resistance to clindamycin, despite the similarity in the target structure of these antimicrobial agents.

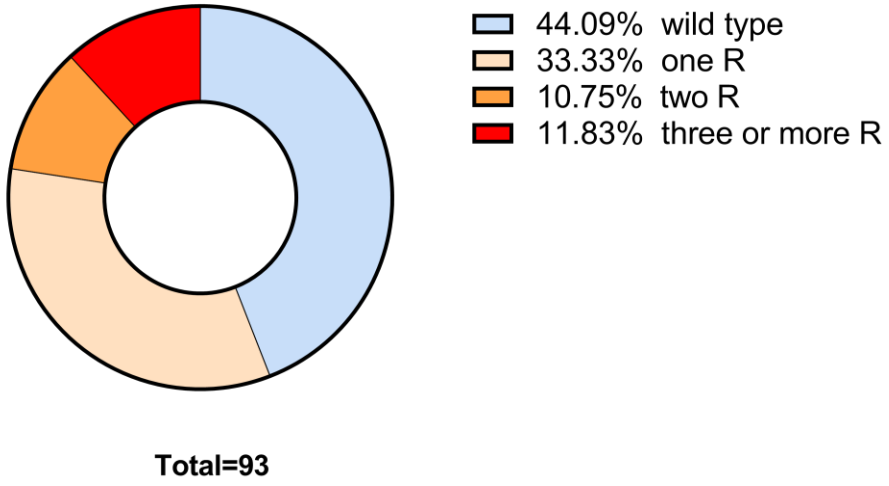

Figure 3: Proportion of antimicrobial resistance phenotypes in *Bacillus cereus sensu lato* from patient samples. The isolates (n = 93) were obtained from microbiological routine diagnostics of patient samples, regardless of their relevance to the disease and including putative sample contamination. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints. The following eight antimicrobials were included: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Isolates without (acquired) resistances were defined as wild type.

Patient isolates – cranial orthosis trial

In the largest subset of isolates, which was obtained from patients enrolled in a clinical trial on helmet molding therapy, the phylogenetic group III predominated substantially (Figure 4). The isolates were obtained from 48 different patients at different time points during the therapy. A total of 132 out of 138 isolates (95.65%) could be assigned to group III comprising *Bacillus mosaicus*. Two isolates each belonged to groups II (*Bacillus mosacius*/*luti*) and V (*Bacillus toyonensis*). Moreover, one isolate each was classified as group IV and group VI, indicating *Bacillus cereus sensu stricto* and *Bacillus mycoides*/*paramycoides*, respectively. Thus, the proportion of phylogenetic groups observed in this subset of patient-derived isolates differs considerably from the distribution observed in isolates from patient samples in routine microbiological diagnostics. Given that multiple isolates were obtained from each of the 41

patients in the course of helmet therapy, the presence of clonal isolates is probable. No mismatch between the seven-group *panC* scheme and the adjusted eight-group framework was identified.

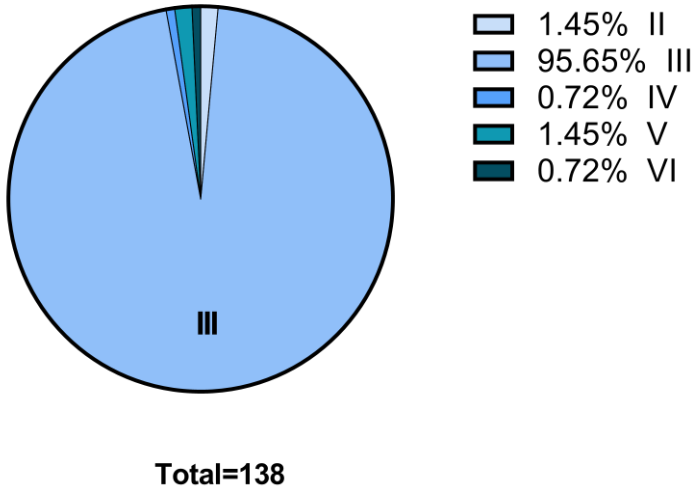

Figure 4: Phylogenetic distribution of *Bacillus cereus sensu lato* from cranial orthoses and the scalp of infants. The isolates were obtained during a clinical trial on helmet molding therapy and were isolated from 48 patients at different time points during the therapy. Phylogenetic assignment of the isolates (n = 138) was based on the *panC* gene sequence. Both, the seven-group and the eight-group adjusted *panC* schemes were used for assignment. (2,44)

The antimicrobial susceptibility of the isolates from cranial orthoses and the scalp of infants also differed from the other isolates from patient samples (Table 3). Of the isolates examined, only six (4.35%) exhibited resistance to erythromycin, which is considerably lower than the number of resistant isolates in samples from patient diagnostics (33.33%). A lower number of resistant isolates was also reported for vancomycin (two isolates, 1.45%). The proportion of isolates resistant to carbapenems was comparable to that observed in other patient samples, with 21 (15.22%) and 35 (25.36%) of the isolates being resistant to imipenem and meropenem, respectively. Resistant phenotypes to ciprofloxacin and levofloxacin were each reported once, but not in the same isolate. Both isolates, however, demonstrated zone diameters of 22 mm, which is close to the clinical breakpoint of 23 mm. Furthermore, all non-resistant isolates were

indicated as “susceptible, increased exposure” to ciprofloxacin and levofloxacin in accordance with the EUCAST clinical breakpoints. All isolates were susceptible to clindamycin and linezolid.

Table 3: Antimicrobial susceptibility of 138 isolates of *Bacillus cereus sensu lato* from cranial orthoses and the scalp of infants. The isolates were obtained during a clinical trial on helmet molding therapy and were isolated from 48 patients at different time points during therapy. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints.

	susceptible	**susceptible, increased exposure**	**resistant**
clindamycin	138	-	0 (0.00%)
erythromycin	132	-	6 (4.35%)
ciprofloxacin	0	137	1 (0.72%)
levofloxacin	0	137	1 (0.72%)
vancomycin	136	-	2 (1.45%)
linezolid	138	-	0 (0.00%)
imipenem	117	-	21 (15.22%)
meropenem	103	-	35 (25.36%)

In accordance with the lower detection of antimicrobial resistances in isolates from cranial orthoses and the scalps, most isolates (63.04%) were classified as wild type strains exhibiting no resistance to any of the clinically relevant antibiotics (Figure 5). A total of 40 isolates (28.99%) were reported to be resistant to only one of the antimicrobial agents tested. Nine of the isolates (6.52%) demonstrated resistant phenotypes to two different antimicrobial agents, while only two isolates (1.45%) were resistant to three or more antimicrobials. Thus, the number of isolates being resistant to at least three isolates is considerably lower than in samples from microbiological patient diagnostics. Notably, one isolate was resistant to five out of eight antimicrobial agents in disk diffusion testing (erythromycin, ciprofloxacin, vancomycin, meropenem, imipenem).

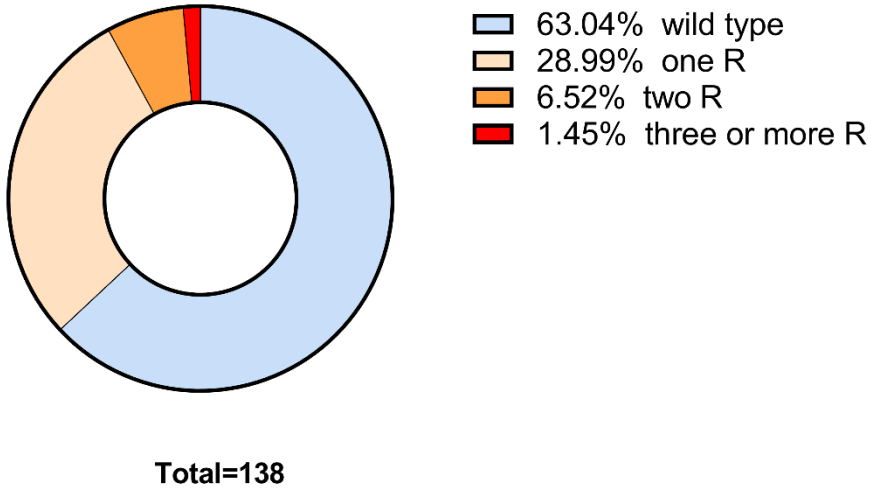

Figure 5: Proportion of antimicrobial resistance phenotypes in *Bacillus cereus sensu lato* from cranial orthoses and the scalp of infants. The isolates (n = 138) were obtained during a clinical trial on helmet molding therapy and were isolated from 48 patients at different time points during therapy. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints. The following eight antimicrobials were included: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Strains without (acquired) resistances were defined as wild type.

Hospital isolates – outpatient clinic

A total of 16 isolates were collected in the outpatient clinic, where those patients, who were enrolled in the clinical trial on helmet molding therapy, were examined. All isolates were assigned to phylogenetic group III, reflecting the distribution of *Bacillus cereus sensu lato* from cranial orthoses. The phylogenetic affiliation was validated by applying both the seven-group and the adjusted eight-group *panC* framework. The observed antimicrobial resistances in the disk diffusion tests were limited to meropenem (Table 4). It is crucial to note that the zone diameters observed below the breakpoint were all attributed to single colonies growing within the inhibition zone of meropenem. No resistant phenotypes were detected for clindamycin, erythromycin, ciprofloxacin, levofloxacin, vancomycin, linezolid and imipenem. All isolates were

classified as “susceptible, increased exposure” for the quinolone antibiotics according to EUCAST clinical breakpoints.

Table 4: Antimicrobial susceptibility of 16 isolates of *Bacillus cereus sensu lato* from the hospital environment. Isolates were collected from various surfaces in the outpatient clinic environment involved in the clinical trial on helmet molding therapy at the Division of Oral and Maxillofacial Surgery. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints.

	susceptible	**susceptible, increased exposure**	**resistant**
clindamycin	16	-	0 (0.00%)
erythromycin	16	-	0 (0.00%)
ciprofloxacin	0	16	0 (0.00%)
levofloxacin	0	16	0 (0.00%)
vancomycin	16	-	0 (0.00%)
linezolid	16	-	0 (0.00%)
imipenem	16	-	0 (0.00%)
meropenem	13	-	3 (18.75%)

As the only detected resistances in disk diffusion tests were reported for meropenem, most of the isolates (81.25%) were classified as wild type (Figure 6).

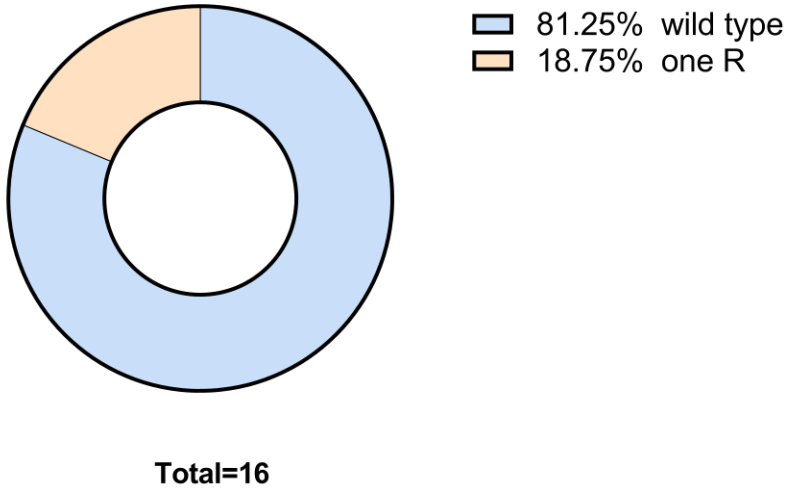

Figure 6: Proportion of antimicrobial resistance phenotypes in *Bacillus cereus sensu lato* from the hospital environment. The isolates (n = 16) were collected in the outpatient clinic environment involved in the clinical trial on helmet molding therapy at the Division of Oral and Maxillofacial Surgery. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints. The following eight antimicrobials were included: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Strains without (acquired) resistances were defined as wild type.

Industrial isolates – food packaging materials

Industrial isolates from the food packaging sector were selected as a reference for the human-associated isolates from patients and from the hospital environment. Most of these industrial isolates (n=80) were obtained from the food packaging materials itself. A large part of the data from Schmid et al. (138) was thereby reanalyzed using both, the seven-group and the eight-group adjusted *panC* schemes. Four different phylogenetic groups were identified with group III being the most abundant among the isolates. (138) In detail, 39 isolates (48.75%) were classified as group III (*Bacillus mosacius*), followed by 32 isolates (40.00%) that belonged to group IV (*Bacillus cereus sensu stricto*) and eight isolates (10.00%) of group II (B*acillus mosacius*/*luti*) (Figure 7). A single isolate of group VI, representing *Bacillus mycoides* and

Bacillus paramycoides, was also identified. All results for groups II, III and VI from the seven-group scheme were confirmed with the Btyper3 software.

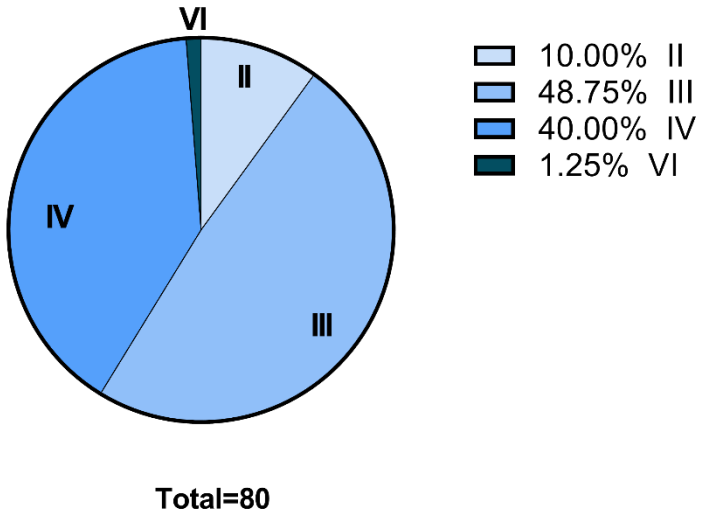

Figure 7: Phylogenetic distribution of *Bacillus cereus sensu lato* from food packaging materials. The isolates were obtained from 41 different food packaging samples. Phylogenetic assignment of the isolates (n = 80) was based on the *panC* gene sequence. The data from Schmid et al. (138) was reanalyzed using both, the seven-group and the eight-group adjusted *panC* schemes (2,44).

A variety of different resistance phenotypes was detected in the food packaging isolates (Table 5). Similar to the patient isolates, resistance to erythromycin was the most prevalent. A total of 20 isolates were classified as resistant to erythromycin, followed by resistance to imipenem and meropenem in 12 (15.00%) and 10 (12.50%) isolates, respectively. The carbapenem resistance in several isolates was again mostly due to single colonies growing in the inhibition zone and therefore leading to a reduced zone diameter. Regarding vancomycin, six isolates (7.50%) were reported resistant. One isolate, BC70, was of particular interest as almost no inhibition zone around the vancomycin disk could be observed. High susceptibility rates were observed for clindamycin (two resistant isolates) and ciprofloxacin (two resistant isolates) as well as for levofloxacin and linezolid (both 100% susceptibility). The isolates classified as resistant to

ciprofloxacin and clindamycin were close to the breakpoints of 23 mm and 17 mm with zone diameters of 22 mm and 16 mm, respectively.

Table 5: Antimicrobial susceptibility of 80 isolates of *Bacillus cereus sensu lato* from food packaging materials. The isolates were obtained from 41 different food packaging samples. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints.

	susceptible	**susceptible, increased exposure**	**resistant**
clindamycin	78	-	2 (2.50%)
erythromycin	60	-	20 (25.00%)
ciprofloxacin	0	78	2 (2.50%)
levofloxacin	0	80	0 (0.00%)
vancomycin	74	-	6 (7.50%)
linezolid	80	-	0 (0.00%)
imipenem	68	-	12 (15.00%)
meropenem	70	-	10 (12.50%)

The majority of the food packaging isolates (55.00%) were susceptible to all tested antimicrobial agents and therefore classified as wild type (Figure 8). Resistance to one of the antimicrobial agents was detected in 21 isolates (26.25%), while 14 isolates (17.50%) exhibited resistance to two different antimicrobial agents in the disk diffusion tests. Only one isolate was reported to be resistant to three agents, namely erythromycin, vancomycin and meropenem.

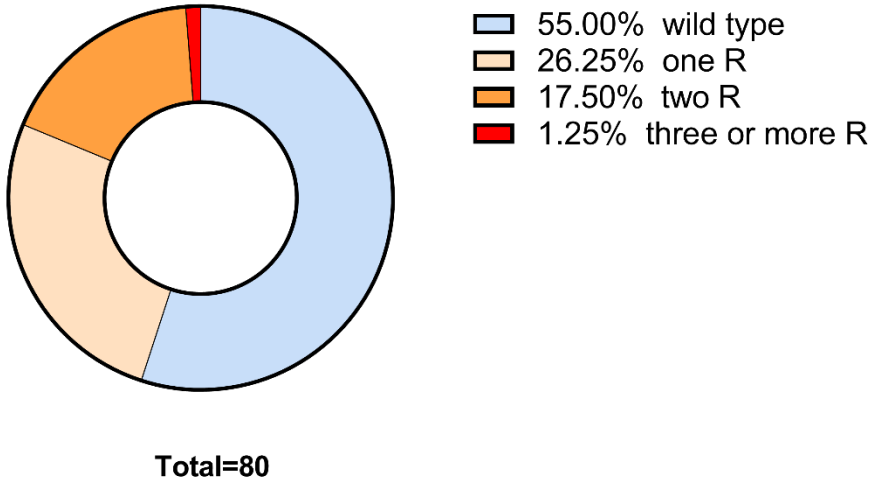

Figure 8: Proportion of antimicrobial resistance phenotypes in *Bacillus cereus sensu lato* from food packaging materials. The isolates (n = 80) were obtained from 41 different food packaging samples. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints. The following eight antimicrobials were included: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Strains without (acquired) resistances were defined as wild type.

Industrial isolates – biofilm and process water

In addition to the food packaging isolates, 26 *Bacillus cereus sensu lato* isolates were obtained from a production site of paper and cardboard associated with the production of a subset of the food packaging samples. A total of four phylogenetic groups were identified among the isolates, with group IV being the most abundant (Figure 9). In detail, 13 isolates were assigned to group IV (*Bacillus cereus sensu stricto*), followed by six isolates of group III (*Bacillus mosacius*), four of group V (*Bacillus toyonensis*), and two of group II (*Bacillus mosacius*/*luti*). One isolate could not be assigned to any of the phylogenetic groups as the PCR amplification of the *panC* gene was unsuccessful when using primers for groups I–VI and for group VII. Abundance of *panC* group IV, however, might be biased due to the isolation of six strains from a single biofilm sample, all of which were identified as belonging to group IV. The possibility of clonal isolates cannot be discounted.

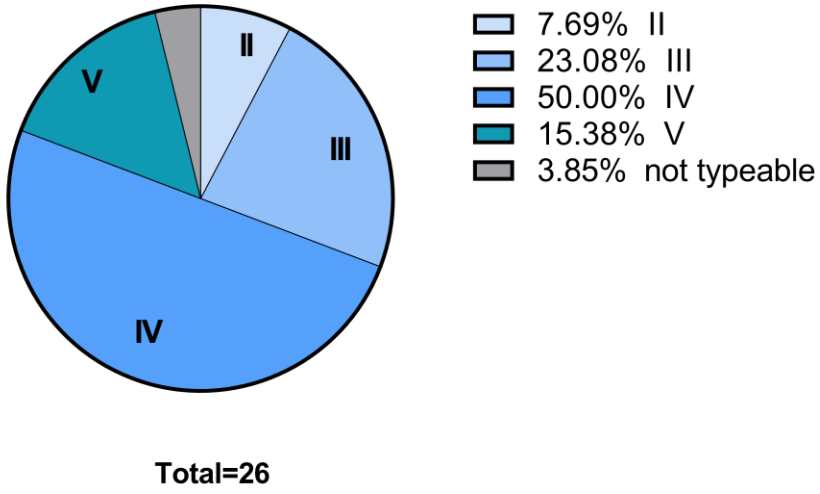

Figure 9: Phylogenetic distribution of *Bacillus cereus sensu lato* in industrial biofilms and process water. The isolates were obtained from seven biofilm samples and one process water sample of a production site of food packaging materials. Phylogenetic assignment of the isolates (n = 25) was based on the *panC* gene sequence. Both, the seven-group and the eight-group adjusted *panC* schemes (2,44) were used for assignment. For one isolate, the *panC* gene could not be amplified via PCR and was therefore classified as not typeable.

Antimicrobial resistant strains were rarely detected in the biofilm and process water population. All isolates were reported as susceptible to clindamycin, vancomycin and linezolid, as well as “susceptible, increased exposure” for ciprofloxacin and levofloxacin (Table 6). A single isolate showed resistance to three of the tested antimicrobial agents (Figure 10). It was reported as resistant to erythromycin, imipenem and meropenem.

Table 6: Antimicrobial susceptibility of 26 isolates of *Bacillus cereus sensu lato* from industrial biofilms and process water. The samples were obtained from a production site engaged in the manufacturing of food packaging materials. The susceptibility testing was

conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints.

	susceptible	susceptible, increased exposure	resistant
clindamycin	26	-	0 (0.00%)
erythromycin	25	-	1 (3.85%)
ciprofloxacin	0	26	0 (0.00%)
levofloxacin	0	26	0 (0.00%)
vancomycin	26	-	0 (0.00%)
linezolid	26	-	0 (0.00%)
imipenem	25	-	1 (3.85%)
meropenem	25	-	1 (3.85%)

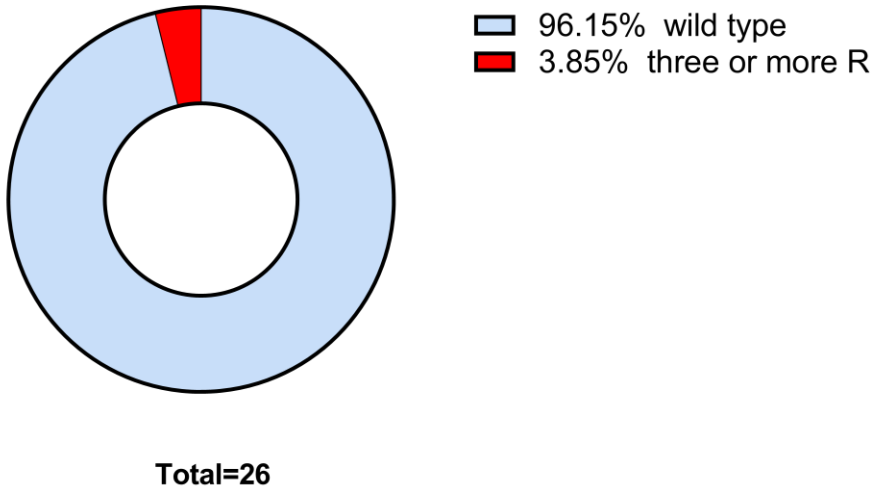

Figure 10: Proportion of antimicrobial resistance phenotypes in *Bacillus cereus sensu lato* from industrial biofilms and process water. The isolates (n = 26) were obtained from seven biofilm samples and one process water sample collected at a manufacturing site for food packaging materials. The susceptibility testing was conducted using the disk diffusion method and in accordance with the current EUCAST guidelines and clinical breakpoints. The following eight antimicrobials were included: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Strains without (acquired) resistances were defined as wild type.

Statistical analysis of patient isolates and environmental isolates

The phylogenetic diversity was assessed between patient isolates from microbiological routine diagnostics (n = 93) and isolates from food packaging materials (n = 80) by comparing the frequencies of phylogenetic groups in each population. To this end, a chi-square test of independence was utilized. The null hypothesis posits that there is statistical independence between the variables in question, namely the isolate origin and the phylogenetic group (II, III, IV, V, VI). However, the expected values for each cell in the contingency table did not meet the requirements of the chi-square test, as both expected values for group VI were below 1.0. Therefore, the values for group V and group VI were merged to satisfy the requisite conditions, creating the variable phylogenetic groups V+VI. The chi-square test demonstrated a statistically significant dependence between the variables, χ^2 (3) = 14.10, P = 0.0028, and the null hypothesis was rejected. As a result, the frequencies of phylogenetic groups were related on the sample origin. As can be discerned from the pie charts, there is a greater evenness in the distribution of phylogenetic groups in patient isolates than in food packaging isolates (Figures 2 and 7). The food packaging materials exhibited a higher concentration of isolates belonging to groups III and IV, whereas groups II and V demonstrated a greater prevalence in the patient isolates. Since clonal isolates could not be discounted in isolates, which were obtained from cranial orthoses and infant scalps, as well as in the biofilm and process water isolates, the decision was made not to include them in the statistical comparison between patient isolates and environmental isolates. This decision was also supported by the results of *panC* typing. The isolates from positive blood cultures covered the groups II, III, IV and V.

A statistical comparison was also conducted to examine the frequencies of antimicrobial resistance in patient isolates and those in environmental isolates. Initially, a chi-square test of independence was used to compare antimicrobial resistant phenotypes in patient isolates from microbiological routine diagnostics and isolates from food packaging materials. The null hypothesis posits that there is statistical independence between the isolate origin and the resistance phenotype. The antimicrobial-resistant phenotypes were classified into four categories for each population (patients and packaging material): wild type, one resistance, two resistances, and three or more resistances. The expected values for each cell in the contingency table exceeded 5, allowing the chi-square test. The chi-square test demonstrated a statistical dependence between the variables, χ^2 (3) = 10.11, P = 0.0177, and the null hypothesis was rejected. The frequencies of antimicrobial resistant phenotypes (wild type, one resistance, two

resistances, and three or more resistances) were therefore related to the source of the isolates. Given the lower number of wild type isolates in the population obtained from patients together with higher frequencies of isolates with one and three or more resistances, the results indicate a higher burden of antimicrobial resistances in isolates from microbiological patient diagnostics than in packaging material isolates (Figure 11).

In addition, a statistical analysis was conducted for all patient isolates consisting of those from routine patient diagnostics as well as those isolated from cranial orthoses and infant scalps. This totalized population was compared with environmental isolates of food packaging material and isolates from biofilms and process water associated. The isolates, which were collected at the outpatient clinic, were not included in the analysis on the grounds that they cannot be considered true environmental isolates, as a human origin from patients was probable. The chi-square test yielded the same result, indicating a statistically significant dependency between the variables, χ^2 (3) = 8.468, P = 0.0373, and the null hypothesis was rejected. Although the frequencies of antimicrobial resistant phenotypes are still statistically attributable to the origin of the isolates, the level significance decreased, when isolates from cranial orthoses and biofilms were included. The decreased level of significance corresponds with the possibility of clonal isolates within the subsets from biofilms and orthoses, potentially introducing a bias in the comparison. The expected values for each cell in the contingency table are greater than 5, with the exception of a single cell (environmental isolates – three or more resistances), where the value is 4.72.

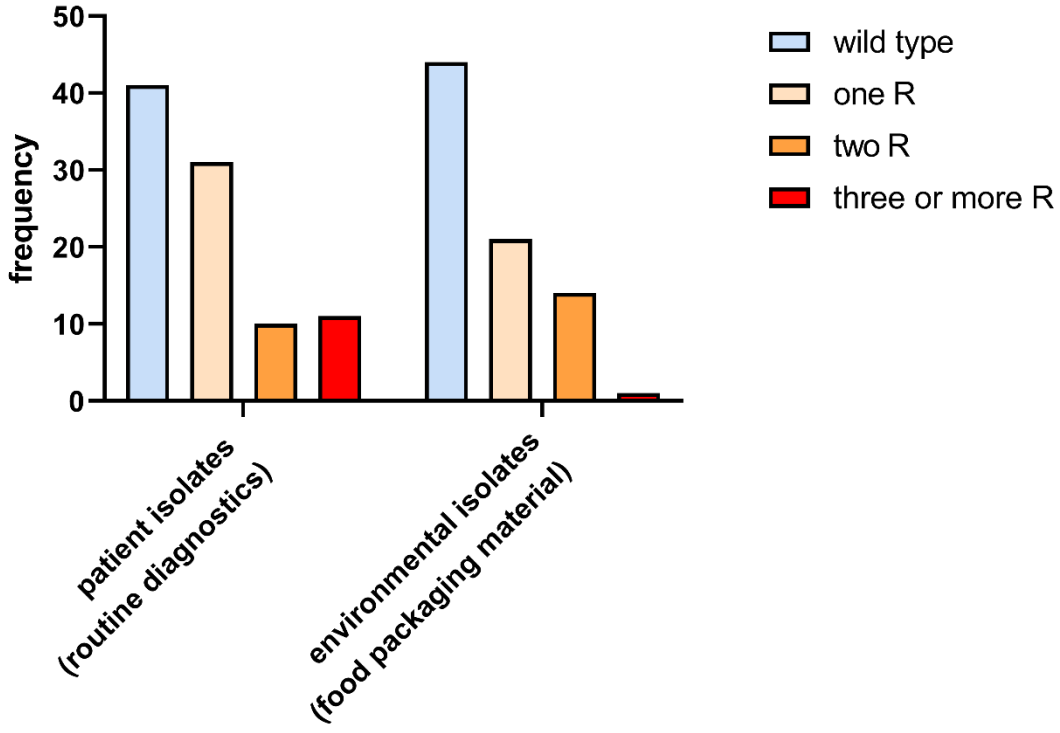

Figure 11: Proportion of antimicrobial resistance phenotypes in patient isolates and environmental isolates. Patient isolates (n = 93) were obtained from microbiological routine diagnostics of patient samples, while environmental isolates (n = 80) were obtained from food packaging materials. The following eight antimicrobials were included in disk diffusion testing: CLI, ERY, CIP, LEV, VAN, LIN, IMI, MER. Strains without (acquired) resistances were defined as wild type.

The frequencies of resistances to individual antimicrobial agents were statistically compared for the patient isolates and the environmental isolates. To minimize the effect of putative clonal isolates, only patient isolates from microbiological routine diagnostics and isolates from food packaging materials were selected. The Fisher's exact test for each comparison did not yield to any statistically significant result (Table 7). The null hypothesis, which stated that there is no association between the isolate's origin and its susceptibility to an antimicrobial agent, was thus not rejected. There was no statistically significant accumulation of certain antibiotic resistances in patient or environmental isolates. This finding suggests that the higher prevalence of

antimicrobial resistance observed in patient isolates is not attributable to resistance to a specific antimicrobial agent in disk diffusion testing.

Table 7: Frequencies of antimicrobial resistances in patient isolates and environmental isolates. Patient isolates were limited to those from microbiological routine diagnostics, while environmental isolates only included those from food packaging materials. Statistical significance ($P < 0.05$) was tested using the Fisher's exact test. ns = not significant.

	patient isolates (routine diagnostics)		isolates from food packaging material		P value (Fisher's exact test)	interpretation
	S	**R**	**S**	**R**		
clindamycin	90	3	78	2	>0.9999	ns
erythromycin	62	31	60	20	0.2464	ns
ciprofloxacin *	92	1	78	2	0.5964	ns
levofloxacin *	91	2	80	0	0.4999	ns
vancomycin	83	10	74	6	0.6007	ns
linezolid	92	1	80	0	>0.9999	ns
imipenem	71	22	68	12	0.1813	ns
meropenem	77	16	70	10	0.4042	ns

* ciprofloxacin and levofloxacin: only susceptible increased exposure

A statistical comparison of resistant phenotypes (wild type, one resistance, two resistances, and three or more resistances) between the isolates from positive blood cultures and the remaining patient isolates was not feasible, as the requisite conditions for the chi-square test were not met. The expected values were below 1.0. Although the AST results suggested a higher prevalence of erythromycin resistance in blood culture strains (five out of eight strains), no statistically significant result was obtained. For the other antimicrobial agents, there was also no statistically significant result obtained. Thus, the null hypothesis was not rejected indicating that increased antimicrobial resistance is not related to strains isolated from positive blood cultures.

Reporting of *Bacillus cereus sensu lato* in microbiological routine diagnostics

An evaluation of 87 patient isolates from microbiological routine diagnostics revealed discrepancies between the detected *Bacillus cereus sensu lato* and the reported microbiological findings at the end. The retrospective analysis on the report frequency resulted in 15 cases, in which *Bacillus cereus sensu lato* was reported including the findings of appropriate AST. The antimicrobial susceptibility was indicated with the MIC or an interpretation including R (resistant), I (susceptible increased exposure), and S (susceptible) of the respective antimicrobial agent. In a majority of 44 cases, *Bacillus cereus sensu lato* was mentioned in the microbiological findings but without AST results. In 15 cases, *Bacillus cereus sensu lato* was not referenced at all in the final medical report, despite having been identified in the patient's sample, isolated, and subsequently provided for further analysis as part of this project. For 13 isolates, no information regarding the medical report could be obtained. Semi-quantitative information on the number of bacteria in the sample, if available, was not evaluated. The results indicate that a significant proportion of isolates were considered as sample contamination or background flora, rather than having clinical relevance. A definitive assessment of whether the isolates should have been classified as contaminants or whether AST would have been the appropriate course of action was not done due to restricted access to patient data and clinical expertise. In the microbiological reports, *Bacillus cereus sensu lato* was indicated as “*Bacillus cereus*”, “*Bacillus cereus group*”, “*Bacillus* spp.” or “aerobic sporeformers”. In cases where AST was included in the findings, the organism was consistently specified as "Bacillus cereus". Six patient isolates were not included as they were not randomly selected, but part of a strain collection. All eight isolates from positive blood culture samples, six of them were part of the strain collection, were referenced in the respective microbiological findings including appropriate AST.

Confirmatory tests for vancomycin-resistant isolates

Given the emerging threat of vancomycin resistance on a global scale, the following experiments were designed to elucidate the phenotypic and molecular characteristics of vancomycin resistance observed in *Bacillus cereus sensu lato* through disk diffusion tests. Vancomycin resistance in *Bacillus cereus* strains is unusual and hardly described. Therefore, all isolates, which exhibited a zone diameter below the breakpoint of 10 mm, were subjected to confirmation

tests to verify true vancomycin resistance. A total of 18 isolates from patient samples (routine diagnostics and cranial orthoses) and food packaging materials were classified as resistant to vancomycin upon disk diffusion testing (Table 8). The isolates are distributed across three phylogenetic groups: group III (11 isolates), group IV (4 isolates), and group V (3 isolates). Consequently, no correlation could be established between the isolates and a specific lineage of strains.

The determination of minimal inhibitory concentrations of vancomycin via the gradient test method (Etest®) initially confirmed the results obtained from disk diffusion. All isolates, which were identified as resistant in disk diffusion testing, were also confirmed as resistant when the gradient test method was employed. The MICs ranged from 6 mg/L up to 64 mg/L. Notwithstanding the discrepancy between the breakpoints set forth by EUCAST and CLSI, the MICs of all isolates exceeded the respective breakpoints of 2 mg/L and 4 mg/L. Furthermore, MICs of 12 mg/L or higher indicated clearly resistant phenotypes. Given the rarity of reports on vancomycin resistance, broth microdilution was utilized as gold standard method for MIC determination to confirm the validity of the observed resistance. In contrast to the results from disk diffusion and gradient test, broth microdilution yielded in significantly lower MICs (Table 8). Consequently, all isolates could be classified as susceptible to vancomycin using the more permissive breakpoint of 4 mg/L by CLSI. Moreover, five isolates demonstrated susceptibility when the current breakpoint of 2 mg/L as defined by EUCAST was applied. The observation that most isolates were above the EUCAST-defined breakpoint, yet remained susceptible when assessed using the CLSI breakpoint, suggests the presence of only marginal or low-grade resistance, if any. Furthermore, the MIC determination was repeated for isolates that showed growth only in a single replicate of a concentration or gave unclear results. In total, ten isolates were subjected to repeated broth microdilution in order to obtain reliable results. In the event of uncertainty, the higher concentration was deemed to be the MIC.

Subsequently, the agar dilution method, which is regarded as an alternative reference method for determining the MIC, was performed for each isolate. Since vancomycin is already incorporated into the solid medium at defined concentrations, agar dilution was also used to determine whether the resistance observed in disk diffusion and gradient tests was a result of growth on solid media such as MH agar. In accordance with broth microdilution, the results from agar dilution confirmed the significantly lower MICs compared to agar diffusion assays. All MICs determined by agar dilution ranged from 2 to 4 mg/L. For two isolates, MIC determination via

agar dilution was repeated due to ambiguous results. The isolate BC_helmet_62 initially exhibited a trailing endpoint with several single colonies from 2 to 8 mg/L. Repeated testing finally yielded 2 mg/L as the MIC. A maximum of one single colony was observed at 2 mg/L per technical replicate, which was ignored according to the reading guide. In contrast with the findings from broth microdilution, the most isolates (16 out of 18) demonstrated a MIC of 2 mg/L, with only two isolates exhibiting a MIC of 4 mg/L. For only six isolates, the MICs obtained from broth microdilution and agar dilution were congruent. Agar dilution yielded lower MICs for eleven isolates, while the broth microdilution resulted in a lower MIC for only one isolate. Nevertheless, the MICs of each isolate showed minimal variation, differing by only one two-fold dilution step, if at all. This range is also accepted for target values of quality control strains. (180,181)

Table 8: Vancomycin resistant isolates in disk diffusion and corresponding confirmation tests. The isolates (n = 18) were classified as resistant to vancomycin according to the current EUCAST guidelines and clinical breakpoints for disk diffusion testing (zone diameter < 10 mm). The confirmation tests and reference methods for the determination of the minimal inhibitory concentration were conducted in accordance with the respective EUCAST guideline.

Vancomycin resistant isolates in disk diffusion		**MIC (mg/L)**		
Origin	**Isolate**	**Etest®**	**Broth microdilution**	**Agar dilution**
Patient isolates	5.644	6 mg/L	2 mg/L	2 mg/L
	8659 II a	8 mg/L	4 mg/L	2 mg/L
	112.903	6 mg/L	4 mg/L	2 mg/L
	14220	12 mg/L	2 mg/L	2 mg/L
	J282	12 mg/L	4 mg/L	2 mg/L
	32672ag	6 mg/L	4 mg/L	2 mg/L
	161869	8 mg/L	2 mg/L	2 mg/L
	BK 23/40	12 mg/L	4 mg/L	2 mg/L
	141.009	12 mg/L	4 mg/L	2 mg/L

	24119a	32 mg/L	1 mg/L	2 mg/L
Patient isolates (cranial orthoses)	BC_helmet_62	32 mg/L	4 mg/L	2 mg/L
	BC_helmet_122	6 mg/L	4 mg/L	2 mg/L
Food packaging isolates	BC8	8 mg/L	4 mg/L	2 mg/L
	BC12	12 mg/L	4 mg/L	2 mg/L
	BC17	12 mg/L	4 mg/L	4 mg/L
	BC39	8 mg/L	4 mg/L	2 mg/L
	BC48	8 mg/L	2 mg/L	2 mg/L
	BC70	64 mg/L	4 mg/L	4 mg/L

As a result, the putative resistance to vancomycin was disproved for all isolates, especially when considering the CLSI breakpoint. Furthermore, the pseudo-resistant phenotype was not related to solid growth media, but a consequence of agar diffusion susceptibility testing using disk diffusion or the gradient test method. (179) In parallel, all susceptibility tests were performed on the reference strain ATCC 14579 as a control. The reference strain yielded a susceptible result in the disk diffusion test and MICs of 2 mg/L in the gradient test, broth microdilution and agar dilution. (179)

The subsequent aim was to provide further description of the pseudo-resistant phenotype of *Bacillus cereus sensu lato* in order to ensure reliable antimicrobial susceptibility testing. The food packaging isolate BC70 was of therefore of particular interest, as the vancomycin disc (5 µg) showed effectively no zone of inhibition, and repeated gradient tests for vancomycin revealed MICs of 24 to 64 mg/L (Figure 12, A and B). Consequently, it was selected as a model strain for further experiments on vancomycin susceptibility testing. The mesophilic strain belonged to phylogenetic group IV, defining the isolate as *Bacillus cereus sensu stricto* according to the current nomenclature. (138,179) Both, agar dilution and broth dilution revealed a MIC of 4 mg/L (Figure 12, C and D). The growth curves of BC70 and reference strain ATCC 14579, which were obtained from the broth microdilution, clearly indicated the respective MICs as the lowest concentration of vancomycin without bacterial growth (Figure 13, Figure 14). The last

concentration below the MIC resulted in delayed growth for both the BC70 isolate and the reference strain. Measurable growth started at approximately nine to ten hours post-inoculation but still leading to clearly visible bacterial growth in the wells after the recommended incubation period. For each concentration, all five replicates yielded consistent results.

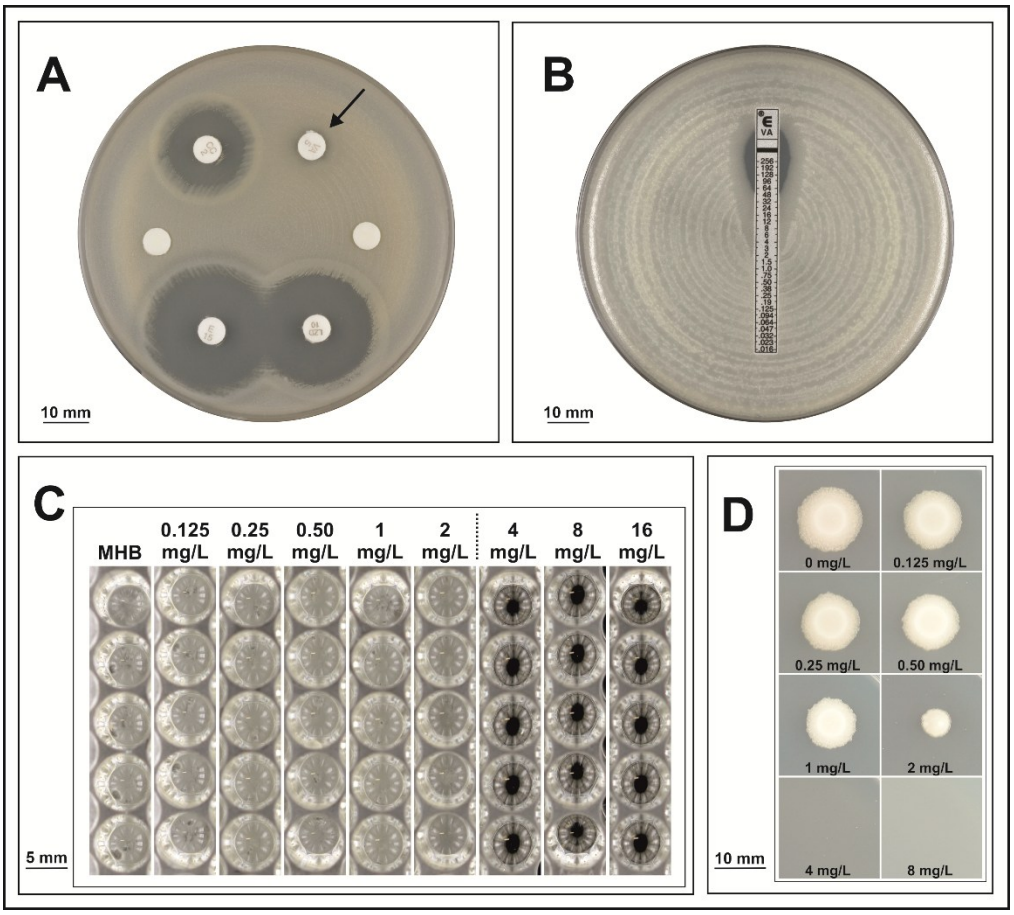

Figure 12: *B. cereus* isolate BC70 displayed pronounced discrepancy in vancomycin susceptibility using different methods. (A) Disk diffusion method, arrow indicates vancomycin disc. (B) Gradient test method with a vancomycin test strip, MIC = 64 mg/L. (C) Broth microdilution, MIC = 4 mg/L. (D) Agar dilution, MIC = 4 mg/L. [Reproduced and adapted from Schmid et al. (179).]

Resistance to vancomycin can concomitantly occur with resistance to other glycopeptides such as teicoplanin. Moreover, the presence of co-resistance to teicoplanin may provide insight into the genetic mechanism of glycopeptide resistance. Therefore, the resistance to teicoplanin in BC70 was evaluated using the gradient test method and revealed a MIC of 4 mg/L, which is

congruent with the results of the reference methods for vancomycin. In summary, the extended phenotypic evaluation of putative resistance to vancomycin could disprove the initially reported resistance by disk diffusion method. In fact, only the reference methods could deliver accurate results on vancomycin susceptibility and testing based on agar diffusion is not reliable. Given that true resistance to vancomycin and teicoplanin could be excluded, subsequent experiments in BC70 were conducted with the objective of elucidating the mechanism that enables the undermining of vancomycin diffusion testing in *Bacillus cereus sensu lato*.

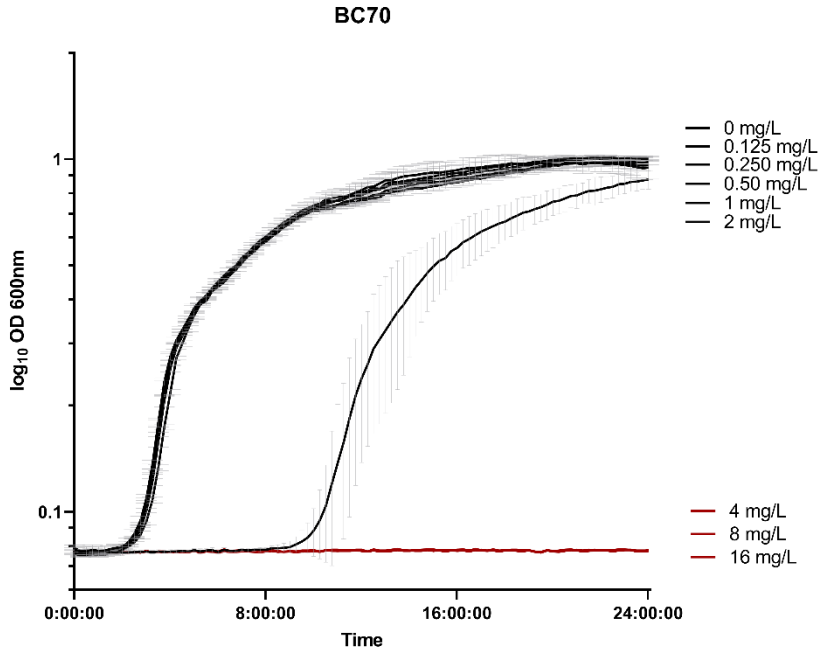

Figure 13: Growth curve for BC70 during broth microdilution. Mueller Hinton broth (MHB) was supplemented with vancomycin ranging from 0.125 mg/L to 16 mg/L. All concentrations were tested in five technical replicates and growth controls without vancomycin (0 mg/L) were included. Growth was quantified by OD_{600} measurement over time. The mean and standard deviation (light grey) are shown on logarithmic y-axis (log_{10}). BC70 showed a MIC of 4 mg/L vancomycin. Inhibitory concentrations of vancomycin resulted in no growth (red lines). The last sub-inhibitory concentration led to delayed growth compared to lower concentrations of vancomycin. [Reproduced and adapted from Schmid et al. (179).]

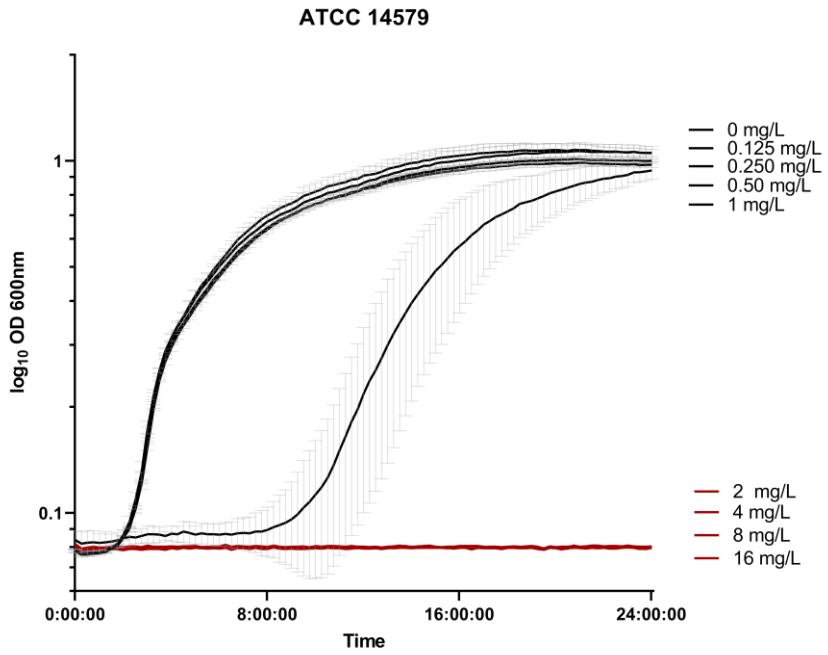

Figure 14: Growth curve for ATCC 14579 during broth microdilution. Mueller Hinton broth (MHB) was supplemented with vancomycin ranging from 0.125 mg/L to 16 mg/L. All concentrations were tested in five technical replicates and growth controls without vancomycin (0 mg/L) were included. Growth was quantified by OD_{600} measurement over time. The mean and standard deviation (light grey) are shown on logarithmic y-axis (log_{10}). ATCC 14579 showed a MIC of 2 mg/L vancomycin. Inhibitory concentrations of vancomycin resulted in no growth (red lines). The last sub-inhibitory concentration led to delayed growth compared to lower concentrations of vancomycin. [Reproduced and adapted from Schmid et al. (179).]

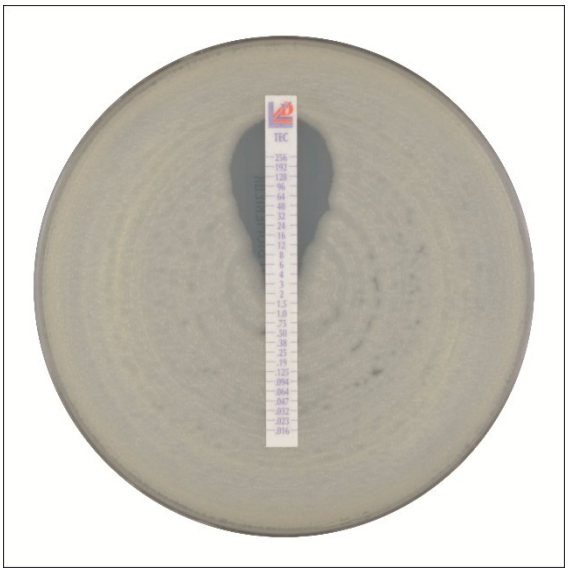

Figure 15: BC70 isolate on MH agar with teicoplanin gradient test. The gradient test method resulted in a MIC of 4 mg/L teicoplanin. There are no breakpoints for teicoplanin specifically for *Bacillus* spp., but breakpoints range from 2 to 4 mg/L for various other species. A standard agar plate with a total diameter of 90 mm (85 mm inner diameter) was used. [Reproduced and adapted from Schmid et al. (179).]

Microscopy of pseudo-resistant isolate BC70

The distinct growth behavior of the pseudo-resistant isolate BC70 was further investigated using different approaches of microscopy. The growth of BC70 when exposed to a vancomycin gradient test (Etest®) was recorded for 24 hours via time-lapse light microscopy. The series of microscopic images demonstrated the proliferation of bacteria up to a MIC of 2 mg/L after a six-hour incubation period (Figure 16), which then continuously expanded in the following hours. After eight hours, a MIC of 3 mg/L was attained, thereby exceeding the EUCAST breakpoint of 2 mg/L for vancomycin. The bacterial expansion on the agar surface persisted and reached a MIC of 24 mg/L after 16 to 20 hours, which is the recommended timeframe for reading the results of agar diffusion susceptibility testing. Prolonged incubation up to 24 hours resulted in a final MIC of 32 mg/L for the gradient test. (179) As previously stated, the final MIC was not consistent and ranged from 24 to 64 mg/L but did not significantly vary after 24 hours. The highest MIC that was observed for BC70 after 48 hours was still 64 mg/L. The initial cell density did not play a decisive role for the migrating phenotype, as comparable MICs were reported for bacterial suspensions

equal to McFarland standards of 0.5 and 1.0 (Figure 17). Microscopic imaging demonstrated that the BC70 isolate was able to migrate over the agar surface into the initial inhibition zone and withstand higher concentrations of vancomycin than the actual MIC. Moreover, the isolate exhibited an impressive speed of translocation covering a distance of 0.5 mm in a single hour. The time-lapse imaging of BC70 was compiled to a movie, which is provided online as supplemental material of the corresponding research paper. (179)

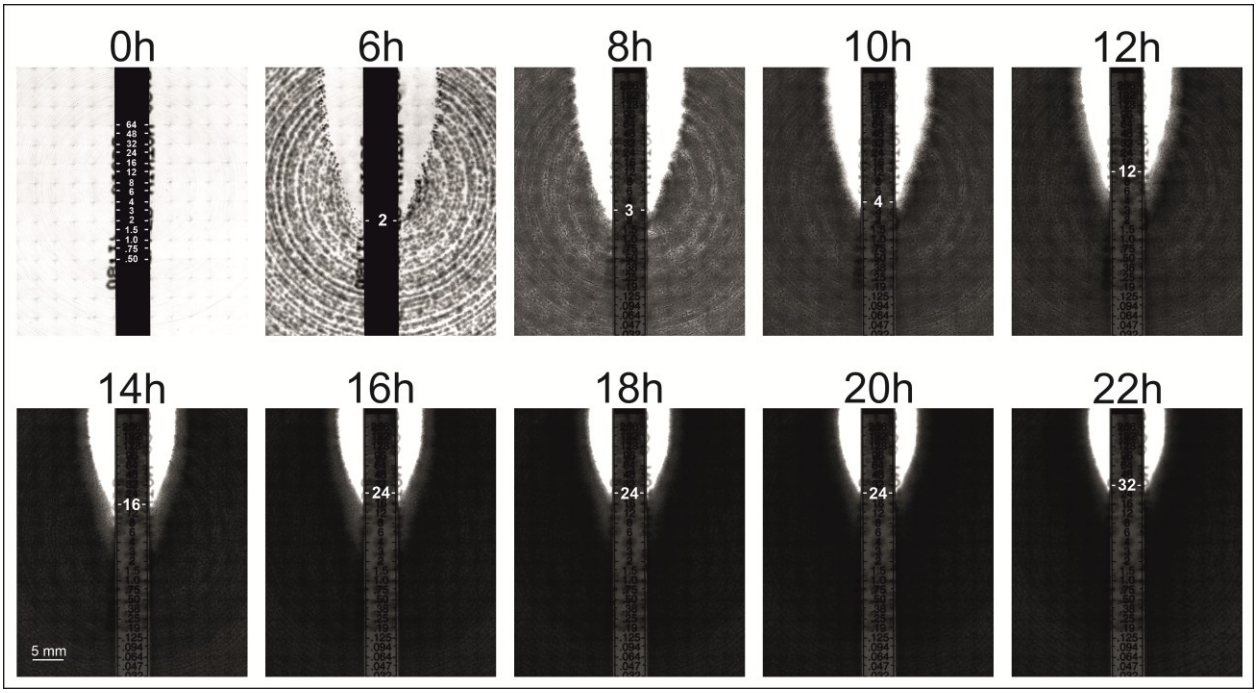

Figure 16: Time-lapse microscopic growth monitoring revealed continuous expansion of BC70 cells on MH agar with vancomycin gradient test. The respective MIC is labelled, starting at 2 mg/L after 6 h and ending at 32 mg/L after 22 h of incubation. The highest point with visible growth along the strip was used to determine the MIC. [Reproduced and adapted from Schmid et al. (179).]

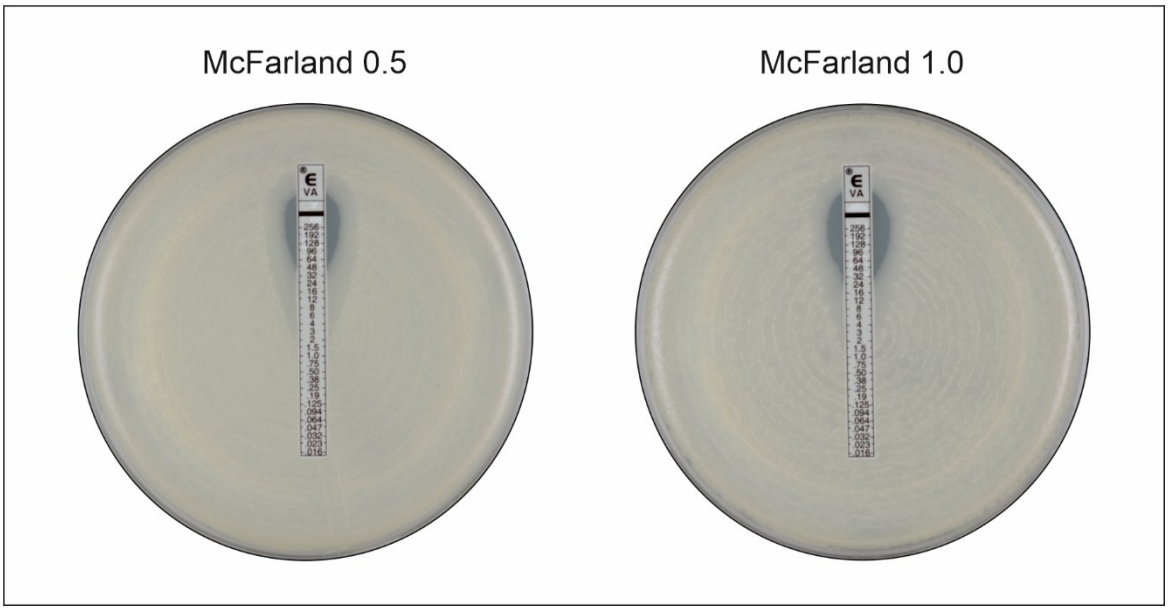

Figure 17: Vancomycin gradient tests (Etest®) for BC70 with different cell densities. Etests® with vancomycin were applied on standard MH plates, which were inoculated with a bacterial suspension equal to McFarland standard of either 0.5 or 1.0. Cell density did not appear to be a crucial factor, as MICs were determined to be 48 mg/L and 32 mg/L for McF 0.5 and 1.0, respectively. Standard agar plates with a total diameter of 90 mm (85 mm inner diameter) were used.

The cells that were migrating into the inhibition zone exhibited a more translucent growth pattern compared to cells situated at the periphery of the gradient test. Due to the larger diffusion area, this translucent growth was easier to detect on plates with gradient tests than on those with disk diffusion. Furthermore, the images of time-lapse microscopy revealed that expanding colonies migrate in a thin layer of advancing cells rather than dense colonies (Figure 18, A). In these images, the cells appeared to follow a tightly aligned orientation and formed an undulating migration front. In response, the colony rims, which were approaching the gradient test, were examined more closely with phase-contrast microscopy. Phase-contrast microscopy permitted an approximate resolution of single cells within the colony rims, demonstrating that the migration front was constituted by tightly aligned, elongated cells that formed finger-like extensions or waves of advancing cells (Figure 18, B and Figure 19, A). In stark contrast, phase contrast microscopic examination of the reference strain ATCC 14579, which did not demonstrate an

advancing phenotype upon agar diffusion testing, revealed neither equally elongated cells nor the formation of aligned and organized multicellular structures at the colony edge towards the inhibition zone (Figure 19, B). The colony edge of ATCC 14579 consisted of short cells, lacking both filamentous growth and uniform orientation. In addition, cells from BC70 were collected from the bacterial lawn, where no inhibitory effects of vancomycin from the gradient test were observed. They were immersed in saline solution and compared with the cells from the colony edge. The BC70 cells in particular displayed a notably elongated phenotype at the border of the inhibition zone, in comparison to the cells from the peripheral bacterial lawn, which were substantially shorter and stocky (Figure 19, C). To eliminate the possibility that immersing the bacterial cells from the periphery into the saline solution resulted in an altered, shorter appearance due to destruction of cellular bundles, the cells at the border of the inhibition zone were also immersed in saline solution and examined microscopically. Since elongated cells were still present after immersing in saline solution (Figure 19, D), the unique phenotype induced at the border of the inhibition zone could be confirmed.

The multicellular, wave-like structures, which were formed by the advancing cells, were shown to be a key feature of sliding motility in *Bacillus subtilis*, and are specifically designated as van Gogh bundles. (186) Given that other bacterial motility mechanisms such as swarming motility can result in similar multicellular structures at colony rims (47), specific staining of the flagella was intended to differentiate between motility mechanisms. Indeed, migrating cells of BC70, which were collected from the migration front after 13 hours of incubation, exhibited a complete absence of flagella (Figure 18, C). Furthermore, the cells that initially appeared to be solely elongated, as observed in phase-contrast microscopy, turned out to be multiple cells arranged in long chains with a clearly visible septation. As a control, swarming cells of BC70, which were obtained from the swarming motility promoting MHS medium, were clearly hyperflagellated and elongated (Figure 18, D). As swarming is regarded as an active motility strategy, it requires the presence of flagella and thus differs from the spreading mechanism observed during vancomycin susceptibility testing, which did not involve an increased use of the flagella. Altogether, the microscopic imaging of BC70 provided compelling evidence that sliding motility is the primary mechanism responsible for migration across the surface. This conclusion was based on the considerable translocation observed, the distinctive van Gogh bundles noted, and the absence of flagella in cells from the border of the inhibition zone.

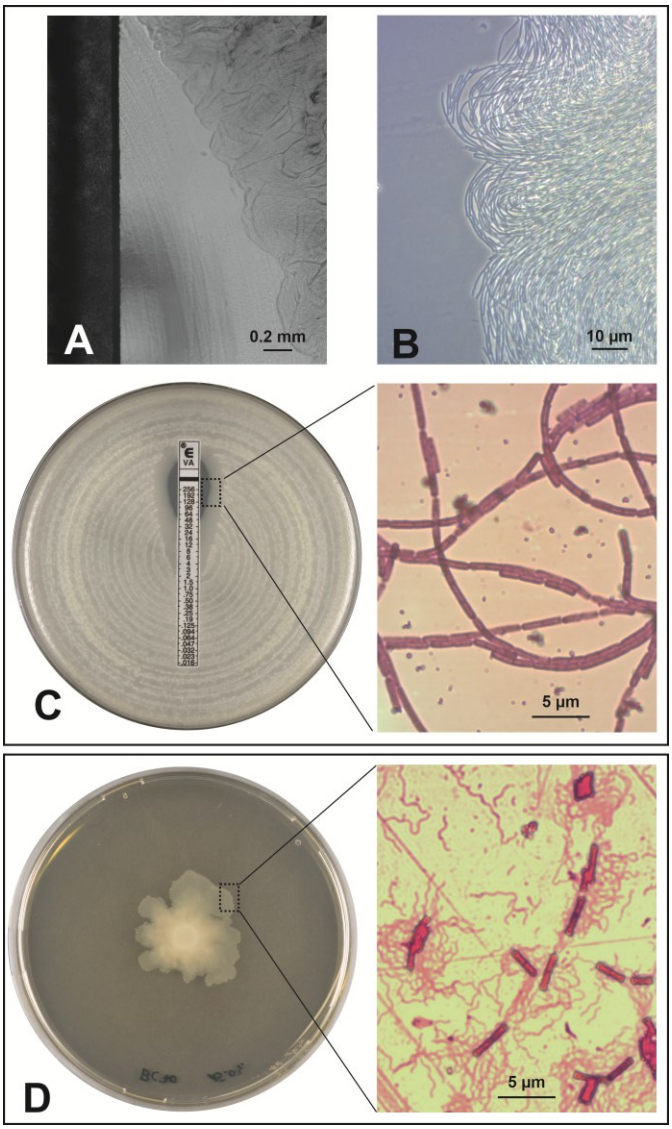

Figure 18: (A) BC70 formed thin layers of cells moving towards the gradient test. (B) Phase-contrast microscopy of BC70 on MH with a vancomycin gradient test showed wave-like colony rim and elongated cell bundles. (C) Flagella stain of BC70 on MH with a vancomycin gradient test disproved swarming motility and revealed long chains of BC70 cells. (D) Distinct swarming phenotype with hyperflagellated cells of BC70 on swarming medium (MHS). [Reproduced and adapted from Schmid et al. (179).]

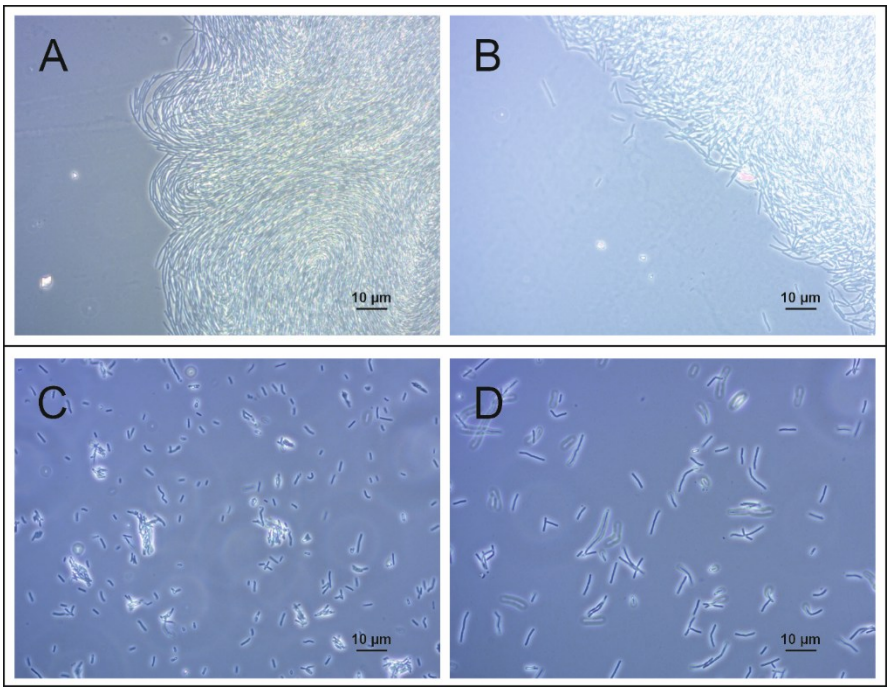

Figure 19: Phase-contrast microscopy of *Bacillus cereus* s.l. cells from MH plates with vancomycin gradient tests. (A) BC70 cells at the border to the inhibition zone display a wave-like morphology with van Gogh bundles. (B) ATCC 14579 did not form van Gogh bundles at the border to the inhibition zone, exhibiting instead diffusely oriented, shorter cells. (C) The BC70 cells from the peripheral areas of the gradient test plates, immersed in 0.9% NaCl solution, exhibited a short, dense phenotype. (D) Elongated cells and multicellular chains of BC70 collected from the border to the inhibition zone, immersed in 0.9% NaCl solution.

Colony expansion assay

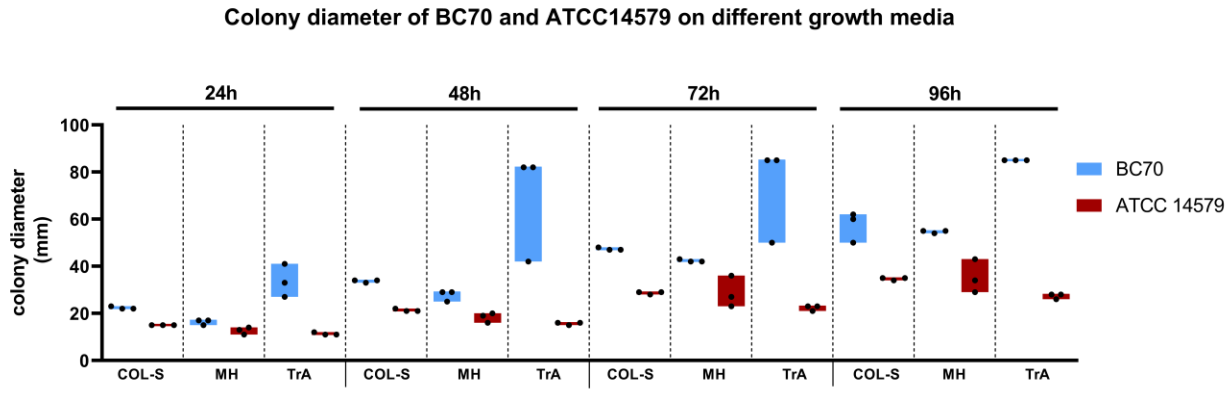

Figure 20: The BC70 isolate showed a strong ability to spread on the agar surfaces of different growth media compared with reference strain ATCC 14579. The following media were used: Columbia agar with 5% sheep blood (COL-S), Mueller Hinton agar (MH) and tryptone swarming agar (TrA). Colony diameters (mm) are shown with boxplots including individual data points. Maximal plate diameter was 85 mm. [Reproduced and adapted from Schmid et al. (179).]

Since the outstanding translocation properties of the BC70 isolate were elucidated by means of different microscopic approaches, the question arose whether its increased mobility is limited to vancomycin susceptibility testing or whether it represents a general feature of this isolate. In a straightforward assay, the expansion capacities of BC70 and the ATCC 14579 were compared on different growth media over time. As colony expansion mainly drives the sliding motility, it can be used as a measure to compare the capacity of sliding motility. Indeed, the BC70 strain demonstrated superior sliding motility compared to ATCC 14579. It exhibited enhanced colony expansion on both Col-S and Mueller Hinton media, while the size of colonies was consistently larger on Col-S than on Mueller Hinton plates. Furthermore, TrA plates, a semi-soft medium containing a reduced agar-agar concentration similar to the MHS medium, was provided to allow swarming motility. The TrA plates yielded the most substantial colony diameters for BC70, though not for ATCC 14579, which demonstrated the smallest expansion on TrA plates. Although BC70 colonies were largest on TrA plates, which should promote swarming motility, they displayed a dendritic morphology instead of continuous swarming (Figure 21, A and B). However, when TrA plates were inoculated with an established swarming population of BC70,

which had been repeatedly pre-cultured on TrA instead of Col-S before inoculation, immediate swarming occurred, resulting in the coverage of the entire plate within 24 hours (Figure 21, C). Based on the results, it could be concluded that BC70 possessed superior mobility on all growth media included in the experiments. Moreover, the motility mechanism in BC70 was found to depend not only on the solidity of the surface. This was evidenced by the observation of different motility phenotypes according to the population used for inoculating the media (swarming population or solid agar grown).

Table 9: Colony diameters of BC70 and ATCC 14579 on different growth media over time. Each measurement was done in triplicates, and the mean diameter is shown (in mm). The range and values of each replicate are illustrated in Figure 19.

	incubation period			
	24 h	**48 h**	**72 h**	**96 h**
Col-S				
BC70	22.33 mm	33.67 mm	47.33 mm	57.33 mm
ATCC 14579	15.00 mm	21.33 mm	28.67 mm	34.66 mm
MH				
BC70	16.33 mm	27.67 mm	42.33 mm	54.66 mm
ATCC 14579	12.67 mm	18.33 mm	28.67 mm	35.33 mm
TrA				
BC70	33.67 mm	68.67 mm	73.33 mm	85 mm
ATCC 14579	11.33 mm	15.67 mm	21.67 mm	26.67 mm

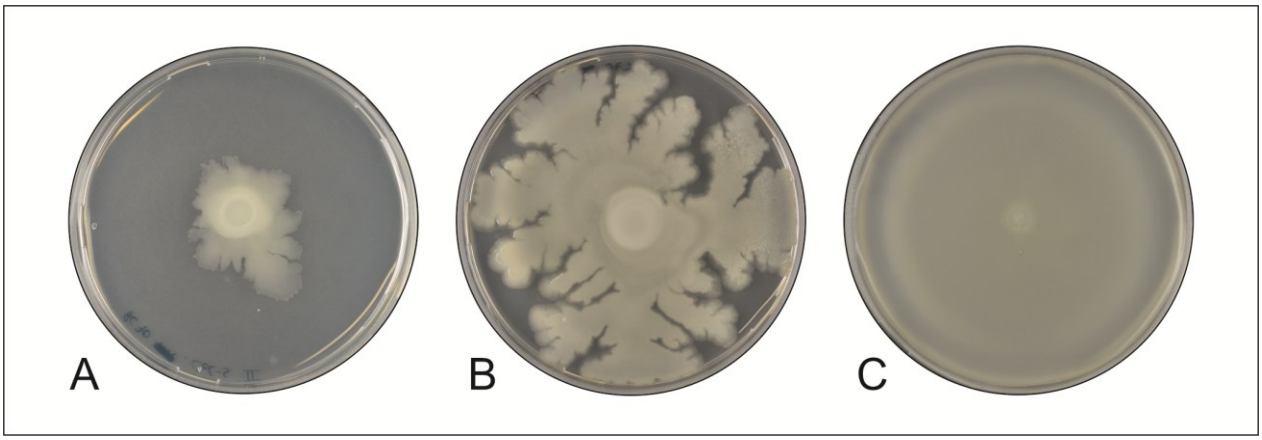

Figure 21: BC70 on TrA swarming agar plates. (A) TrA plate was spot-inoculated with BC70 from Col-S and incubated for 24 h in a humidified chamber. Surface spreading is based on the formation of dendritic colony extensions. (B) BC70 from Col-S spread across the entire TrA plate within 96 h using a dendritic colony morphology. (C) Spot inoculation of pre-established swarming population of BC70 from TrA plates homogenously covered the whole plate by swarming motility within 24 h.

Transcriptomic analysis

The phenotypical description of isolates that appeared to be resistant in agar diffusion testing, in particular the food packaging isolate BC70, led to the assumption of sliding motility as responsible mechanisms for surface spreading during susceptibility testing. In order to confirm the sliding phenotype and to learn more about the observed mobility, RNA-Seq transcriptomic analysis for differential gene expression was performed via nanopore sequencing. The BC70 isolate was selected and compared to the reference strain ATCC 14579. Bacterial cells of both strains were collected from the edges of the respective inhibition zones after exactly 13 h of incubation on plates with a vancomycin gradient test to study actively growing cells during antimicrobial susceptibility testing. (179) Furthermore, BC70 in a swarming state and normal colonies from MH plates were included. The amounts of total RNA extracted from pooled samples prior to ribosomal RNA (rRNA) digestion and library preparation are presented in Table 10.

Table 10: Total RNA extracted from pooled samples prior to rRNA digestion and library preparation. RNA concentrations were determined with the Qubit fluorometer. A total volume of 130 µL was obtained for each pooled sample. In subsequent steps, RNA was not quantified again.

Pooled sample	Extracted total RNA (µg)	Concentration (ng/µL)
BC70 Etest®	2.96	22.8
ATCC 14579 Etest®	3.17	24.4
BC70 swarming	7.79	59.9
BC70 MH colonies	6.64	51.1

Initially, BC70 and ATCC 14579 from gradient test plates were compared to each other. By employing the EPI2ME Transcriptomes workflow, a multitude of differentially expressed genes were successfully identified between BC70 and ATCC 14579 (Figure 22). Further data analysis was performed for differentially expressed genes with a fold change of at least ± 2 and at a statistically significant level with a false discovery rate (FDR) adjusted P value of <0.05. Following these criteria, a total of 164 genes were identified to be differentially expressed between BC70 and ATCC 14579. More specifically, 104 genes in BC70 cells were up-regulated whereas 60 were down-regulated compared with ATCC 14579 cells. Among the differentially expressed genes, a functional classification based on gene ontology revealed 25 significantly overrepresented GO terms (Figure 23). From the perspective of the BC70 isolate, genes associated with pathogenesis, hemolysis and toxin activity were shown to be mostly downregulated whereas sporulation-associated genes were upregulated. Moreover, genes assigned to the GO terms of antibiotic biosynthetic process and glycogen biosynthetic process showed increased expression, as did genes for DNA transposition processes. A total of 18 genes were assigned to the extracellular region, of which nine genes were upregulated and nine genes were downregulated. This indicated relevant changes in transportation and secretion processes, which were involved in mimicking the vancomycin resistance in agar diffusion tests. However, only 63 genes could be assigned to a GO classification. The cluster of orthologous groups (COG) analysis resulted in only two clusters each for upregulated and downregulated genes. For genes that were upregulated, the clusters “METABOLISM; Carbohydrate transport and metabolism” (category G) and “METABOLISM; Lipid transport and metabolism” (category I) were statistically significant. These clusters consisted of six and five assigned genes,

respectively. Among the downregulated genes, the clusters “METABOLISM; Energy production and conversion” (category C) and “METABOLISM; Coenzyme transport and metabolism” (category H) were identified as statistically significant, which consisted of seven and three assigned genes, respectively. (179)

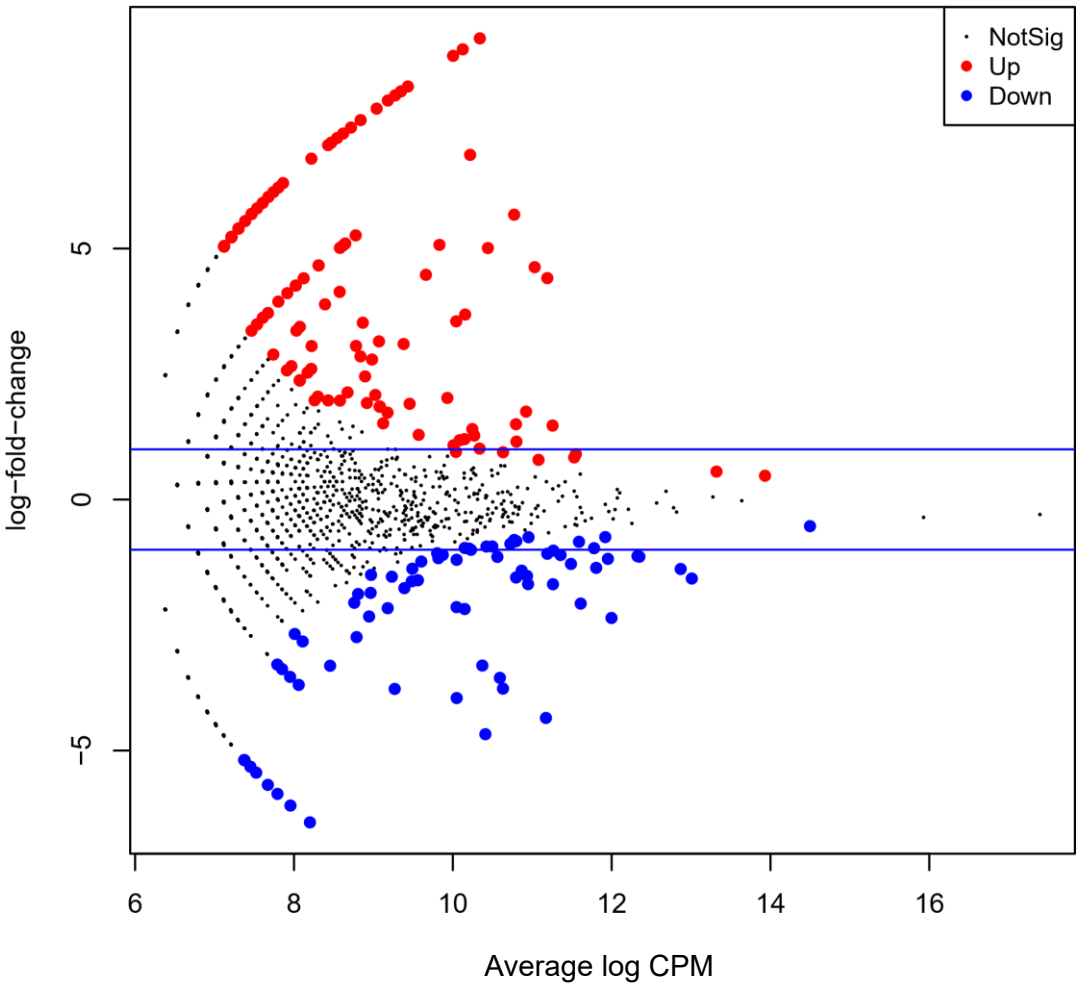

Figure 22: R graphics output from Epi2Me differential gene expression analysis of BC70 and ATCC 14579. Gene expression from cells at the edges of the inhibition zone was compared. The results are shown from the perspective of the BC70 isolate in comparison to ATCC 14579. An overview of the distribution of differentially expressed genes is given. Log-fold change of ± 1 (fold change of ± 2) is indicated with horizontal blue lines. Statistically significant (P value 0.05) genes are highlighted. The x-axis represents the average counts per million of the detected genes.

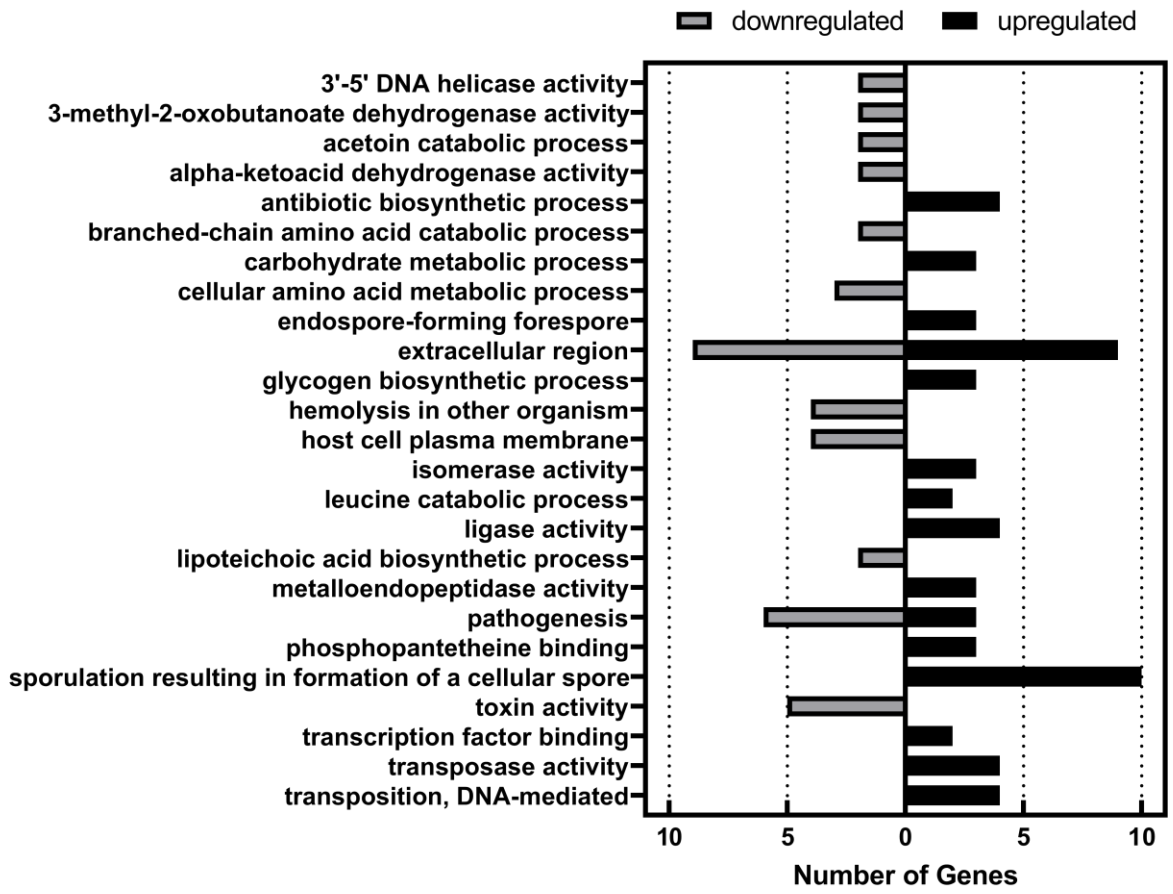

Figure 23: Overrepresented GO terms in BC70 cells with exposure to a vancomycin gradient test. Results are relative to reference strain ATCC 14579, also exposed to a vancomycin gradient test. A total of 63 out of 164 differentially expressed genes could be assigned to a GO term. [Reproduced and adapted from Schmid et al. (179).]

Manual classification of the 164 differentially expressed genes revealed that genes directly involved in biofilm formation or biofilm-associated genes, as well as putative quorum-sensing genes and several sporulation genes were mostly upregulated in BC70 compared to ATCC 14579 (Figure 24). Specifically, two genes whose products play crucial roles as structural components in biofilms were found. Moreover, eight genes previously associated with biofilm formation in *Bacillus cereus sensu lato* were upregulated, while only three genes thought to be associated with biofilms were downregulated. Three of the upregulated genes were identified as being associated with putative quorum sensing systems in the *Bacillus cereus* group, while a

total of 12 upregulated genes were found to be involved in sporulation. Conversely, eight genes that were previously linked to the planktonic growth of *Bacillus cereus* s.l. or identified as being repressed in biofilms were also downregulated in this study. In accordance with the results from GO analysis, virulence-associated genes were mainly downregulated in BC70. In detail, 11 genes were downregulated, while only three of the genes, which were significantly upregulated, were related to virulence. Additionally, ambiguous results were obtained for genes associated with the cell wall biosynthesis and its modification, with two genes upregulated and four genes downregulated, as well as ribosomal genes and protein biosynthesis. The latter indicated that there were no major differences in the metabolic activity between BC70 and ATCC 14579 as protein biosynthesis and ribosomal turnover were similar. In general, genes could be assigned to one or more functional groups. However, the majority of the differentially expressed genes could not be linked to a specific biological function or were only vaguely associated with general metabolic pathways such as those involved in carbohydrate, amino acid or lipid metabolism. (179)

Among the most prominent genes that were differentially expressed between BC70 and ATCC 14579, key genes of biofilm formation and virulence were identified. In BC70, the genes for the biofilm matrix proteins *calY* (FORC47_RS06660/BC1281) and *tasA* (FORC47_RS06650/BC1279) had more than 4- and 21-fold higher transcription, respectively, than in ATCC 14579 cells (Figure 25). The *calY* gene also showed the highest statistical significance of all differentially expressed genes here (FDR adjusted P value = 7.56×10^{-41}). Further upregulated genes clearly associated with biofilm formation were the *krs* operon encoding the lipopeptide kurstakin. The four major genes of the operon, *krsE* (FORC47_RS12230/BC2450), *krsA* (FORC47_RS12235), *krsB* (FORC47_RS12240/BC2453) and *krsC* (FORC47_RS12250/BC2456), showed a 32-fold, 281-fold, 117-fold and 25-fold increased expression in BC70, respectively. Additionally, the biofilm-associated regulators encoded by *mogR* (FORC47_RS08490/BC1655) and *comER* (FORC47_RS22355/BC4325) showed significantly increased expression by factors of 6 and 33**,** respectively. The regulator encoded by *mogR* is a transcriptional repressor of flagellar motility and virulence that promotes biofilm gene expression. (187) The *comER* gene codes for a regulator that plays an important role in biofilm formation in both *Bacillus cereus* and *Bacillus subtilis*. (188) There were also 12 upregulated genes encoding proteins involved in sporulation and its regulation including *spo0M* (FORC47_RS11275/BC2259), *spoIIAA* (FORC47_RS21025/BC4074), *spoIIAB*

(FORC47_RS21020/BC4073), *spoIIE* (FORC47_RS00365/BC0069), *spoIVA* (FORC47_RS07815/BC1509), *spoVG* (FORC47_RS00285/BC0053) and *sigF* (FORC47_RS21015/BC4072). The expression of *spoVG* showed a 2-fold higher transcription and leads to the formation of SpoVG, a crucial regulator in both biofilm formation and sporulation (189). Notably, one gene coding for a flagellin (FORC47_RS08600/ BC1656) was also upregulated in BC70. (179)

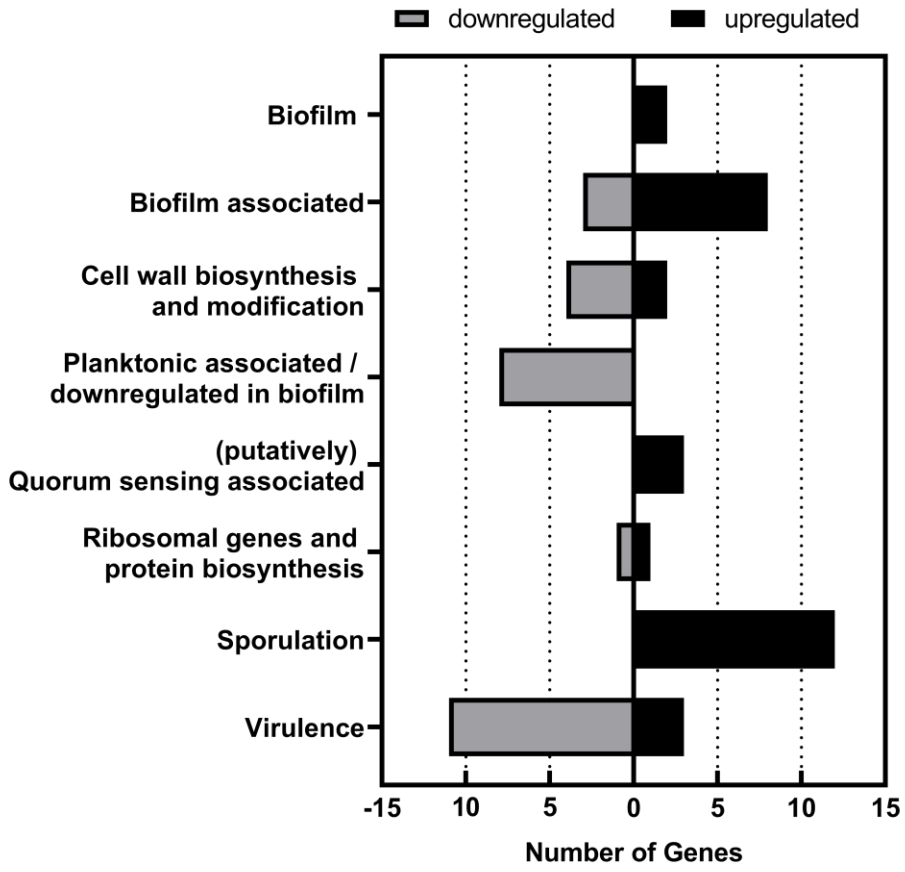

Figure 24: Differentially expressed genes between BC70 and ATCC 14579 clustered according to their biological functions. The differential expression analysis was conducted for the BC70 isolate in comparison to the reference strain ATCC 14579. The genes were manually assigned to their biological functions as proposed in the literature. Only a small proportion of the differentially expressed genes could be successfully assigned. Genes could be assigned to one or more functional groups. [Reproduced and adapted from Schmid et al. (179).]

The genes, which were identified as being downregulated in BC70 in comparison to ATCC 14579, included the most well-characterized enterotoxins of *Bacillus cereus sensu lato*. Both, the entire *nhe* operon (FORC47_RS09385 – RS09395/BC1809 – BC1811) and the entire *hbl* operon were equally downregulated (FORC47_RS12350 – RS12360/BC3104 – BC3102) (Figure 25). The individual toxin-encoding genes exhibited reduced expression levels in BC70, with a range of 10-fold to 26-fold decreases. Furthermore, the expression of cereolysin-encoding gene *clo* (FORC47_RS26330/BC5101) was significantly downregulated as well as other virulence-associated genes such as immune inhibitor *inhA2* (FORC47_RS03525/BC0666) and the neutral proteases *nprP2* (FORC47_RS13955/BC2735) and *nprB* (FORC47_RS27630/BC5351). The metabolism-related genes *pruA*, *rocD*, *hutI* and *gcvPA*, the gene *lldp* coding for L-lactate permease, and the transporter-encoding gene *gltP* as well as some genes encoding proteins of the tricarboxylic acid cycle were downregulated. These genes were previously shown to be associated with planktonic cells or were expressed at decreased levels in biofilms of *Bacillus cereus*. (190) However, some genes that were previously linked to biofilm formation were downregulated, such as *pyrB* involved in pyrimidine metabolism, a collagen adhesion protein (FORC47_RS04615/BC0887), or *dltA* and *dltB* for modification of lipoteichoic acids. (179)

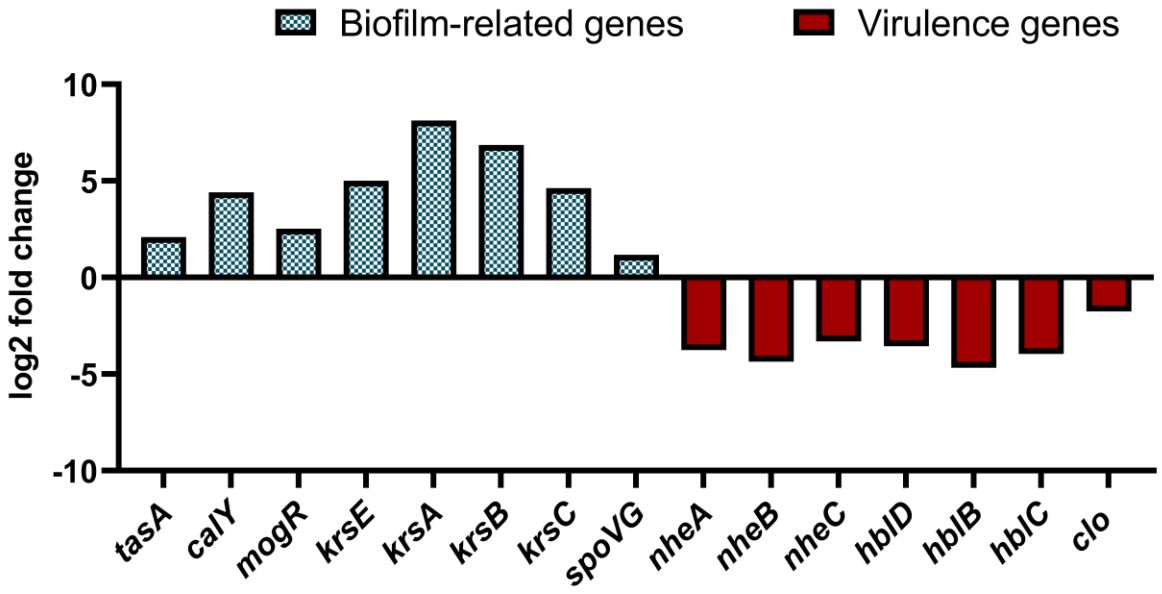

Figure 25: Log2 fold change in gene expression of selected biofilm and virulence genes in BC70 relative to ATCC 14579. [Reproduced and adapted from Schmid et al. (179).]

As a conclusion, the gene expression profile of BC70 cells during vancomycin agar diffusion testing was substantially different from the expression profile of ATCC 14579. The evident phenotypic differences that were observed between the strains could be well-reflected in their respective transcriptome. In order to rule out swarming motility not only by microscopic examination but also at the molecular level, the gene expression of BC70 cells from the border of the inhibition zone were also compared to a swarming population of BC70. Swarming cells were also grown for exactly 13 h on MHS agar to minimize effects arising from nutrient supply or altered incubation period. Following the same criteria for data analysis, a total of 377 differentially expressed genes were successfully identified. Among those genes, 191 were classified as upregulated and 186 genes were classified as downregulated in swarming cells of BC70. Specifically, the swarming cells showed increased expression of 23 genes directly involved in the flagellar system (Figure 26). Furthermore, numerous virulence genes (*hblDAC(B), nheABC,* phospholipases, sphingomyelinase), including the gene for the virulence regulator PlcR (FORC47_RS27625/BC5350), were widely upregulated, whereas sporulation genes and biofilm-affecting genes such as *calY*, *sipW* (FORC47_RS06645/BC1278) and *mogR* were substantially downregulated in swarming cells. The *sinR* (FORC47_RS06665/BC1282) gene, which encodes a master regulator and repressor of biofilm formation, was also expressed at higher levels in the cells from the gradient test than in the swarming cells, a result that is not comprehensible at first glance. Nevertheless, the gene expression profile of swarming BC70 is not only different from the assumed sliding cells, but also clearly identifies the swarming phenotype at the molecular level. A swarming phenotype could therefore be excluded on the basis of phenotypic and molecular traits. (179)

To ascertain whether sliding motility is present in normal BC70 colonies to the same extent as it was on agar diffusion plates, the normal expression profile of BC70 cells on MH agar without any vancomycin was compared with that of BC70 from the gradient test plate (Figure 26). The differential expression analysis revealed a significant upregulation of virulence genes and a simultaneous downregulation of biofilm and sporulation genes in BC70 colonies without vancomycin exposure. From the opposite perspective, the BC70 isolate used a distinct type of cells at the migration front to the inhibition zone, as evidenced by the increased expression of biofilm-associated genes such as *calY*, *sipW*, *krsE*, and *comER* as well as 12 distinct sporulation-related genes. Moreover, five genes could be associated with putative quorum

sensing systems based on a literature review. The MH colonies, in contrast, resembled planktonic cells rather than biofilm or sliding cells. Both the swarming cells and the MH cells showed enhanced expression of protein biosynthesis genes and ribosomal genes, indicating a more active metabolism compared to the cells at the edge of the inhibition zone. (179)

A full list of differentially expressed genes can be found online as supplemental material of the article “Sliding motility of Bacillus cereus mediates vancomycin pseudo-resistance during antimicrobial susceptibility testing”. (179)

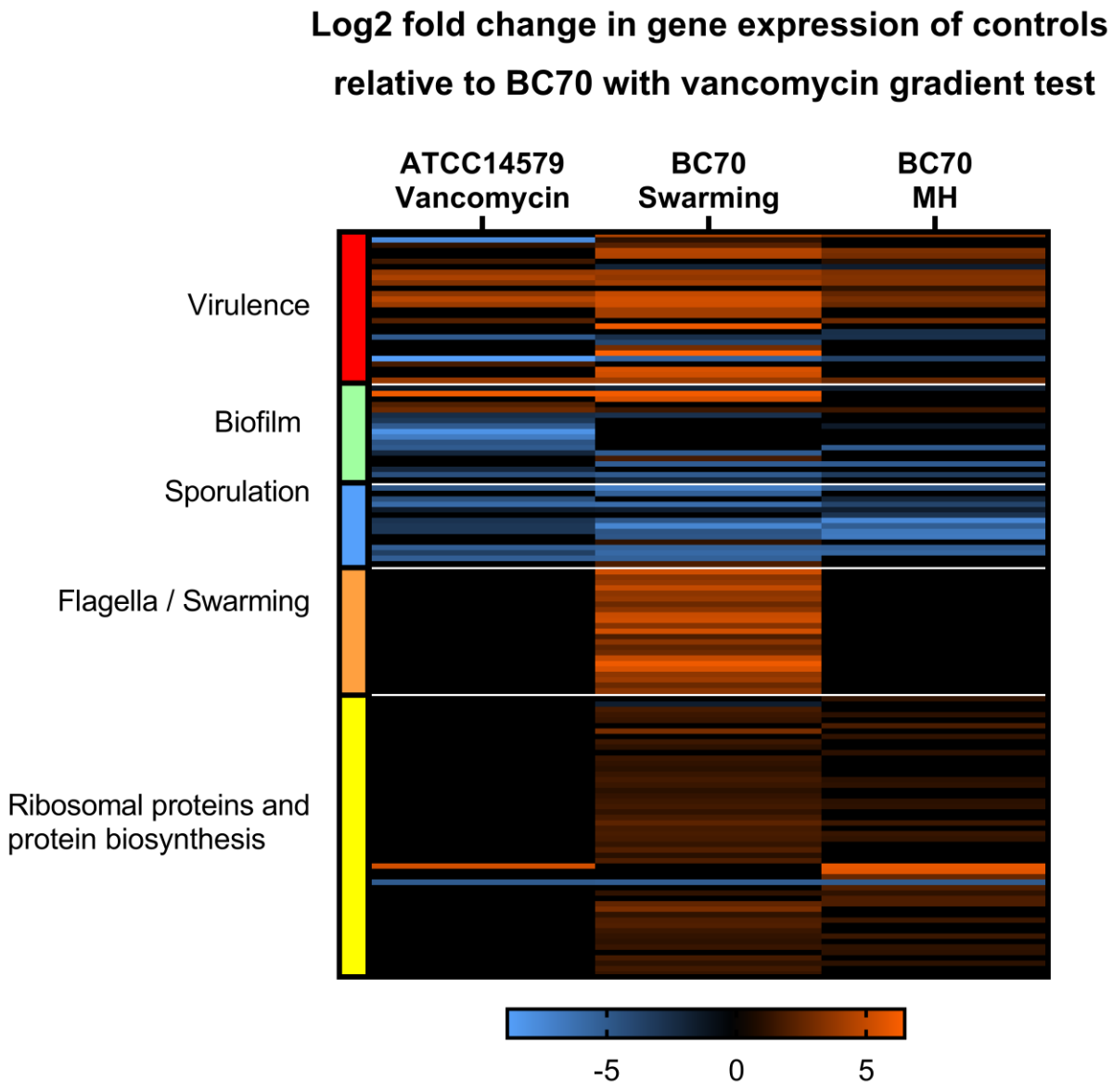

Figure 26: Heat map of differentially expressed genes (log2 fold change). The following conditions were compared: ATCC 14579 with exposure to a vancomycin gradient test, BC70 swarming cells and BC70 on plain MH agar. All conditions were compared with BC70 cells from the border of the inhibition zone (with vancomycin gradient test). Classification of genes according to their biological function was performed manually based on a profound literature review. [Reproduced and adapted from Schmid et al. (179).]

The RNA-Seq results confirmed that BC70 uses a mechanism distinct from swarming motility to spread in the inhibition zone and revealed a biofilm-associated gene expression pattern providing solid evidence to support the sliding motility during susceptibility testing. (179)

Impact of sliding motility on AST of erythromycin and carbapenems

The results of the present thesis highlighted the importance of sliding motility in the susceptibility testing of vancomycin in *Bacillus cereus sensu lato*. The influence of sliding motility on the susceptibility testing of other agents, however, has not been investigated so far. Nevertheless, sliding motility might be affecting erythromycin and carbapenem susceptibility testing as well. Disk diffusion testing revealed that 15 out 18 isolates, which were pseudo-resistant to vancomycin, probably due to sliding motility, were also resistant to erythromycin (Table 11). Although the inhibition zone of erythromycin disks was substantially larger (breakpoint 24 mm) compared to vancomycin, no typical migration front including low cell-density and translucent growth as described for BC70 was observed. Furthermore, the BC70 isolate, which exhibited the most prominent sliding phenotype of all isolates as indicated by the vancomycin MIC of 64 mg/L, was clearly not resistant to erythromycin showing a zone diameter of 28 mm. When looking at vancomycin pseudo-resistance and the susceptibility to carbapenems, conclusions are even more difficult to draw. Only half of the isolates that were pseudo-resistant to vancomycin showed phenotypes putatively resistant to meropenem or imipenem (Table 11). In general, several isolates demonstrated hetero-resistant phenotypes for carbapenems, with the formation of single colonies frequently observed within the inhibition zone, thereby reducing the zone diameter. For a total of 240 isolates (67.99%) out of all 353 isolates included in this study, the reported zone diameters of either meropenem or imipenem or both were affected by the presence of single colonies in the inhibition zone. Specifically, for meropenem, imipenem and both agents, the number of isolates showing blurred edges and single colonies was 153, 205 and 125 isolates, respectively. The single colonies did not necessarily result in zone diameters below the breakpoint. In the subgroup of vancomycin pseudo-resistant strains, this phenomenon was observed for nine out of 18 isolates but did not always coincide with a zone diameter below the breakpoint for the carbapenems. Furthermore, it should be noted that putative heteroresistance to meropenem did not necessarily imply heteroresistance to imipenem, and vice versa.

Table 11: Vancomycin pseudo-resistant isolates and their susceptibility to erythromycin, meropenem and imipenem. Antimicrobial susceptibility testing was performed according to the current EUCAST guidelines and clinical breakpoints for disk diffusion testing.

Origin	Isolate	VAN	ERY	MEM	IMI
Patient isolates	5.644	**R**	**R**	S	S
	8659 II a	**R**	**R**	**R**	**R**
	112.903	**R**	**R**	S	S
	14220	**R**	S	S	**R**
	J282	**R**	**R**	**R**	S
	32672ag	**R**	**R**	S	S
	161869	**R**	**R**	S	S
	BK 23/40	**R**	**R**	S	S
	141.009	**R**	**R**	S	**R**
	24119a	**R**	**R**	**R**	**R**
Patient isolates (cranial orthoses)	BC_helmet_62	**R**	**R**	S	S
	BC_helmet_122	**R**	**R**	**R**	**R**
Food packaging isolates	BC8	**R**	**R**	S	S
	BC12	**R**	**R**	**R**	S
	BC17	**R**	**R**	S	S
	BC39	**R**	**R**	S	S
	BC48	**R**	S	S	**R**
	BC70	**R**	S	S	**R**

Despite incongruent results of vancomycin pseudo-resistance and observed carbapenem resistance, there was also strong evidence that sliding motility indeed affects carbapenem

susceptibility testing. The BC70 isolate did not only show strong sliding capacities on agar diffusion plates with vancomycin, but also a distinct migration front into the inhibition zone for imipenem (Figure 27, A and B). Additionally, single colonies that extended into the meropenem inhibition zone were observed, resembling the sliding morphology. While exposure to imipenem resulted in a zone diameter of 25 mm (breakpoint 30 mm), meropenem yielded 28 mm (breakpoint 25 mm). Although the BC70 cells at the border of the inhibition zone were not examined the same way as those from vancomycin testing, sliding motility represents a plausible cause for the observed phenotypes. The sporadic observation of single colonies growing in the inhibition zone of meropenem lends support to the assumption that this is not genuine resistance (Figure 27, A). Also, a variation of the zone diameters could be observed in repeated tests.

Some isolates were clearly susceptible to vancomycin in disk diffusion testing but displayed highly resistant phenotypes to both meropenem and imipenem, which strongly resembled the aforementioned sliding motility (Figure 27, C). The zone diameters of the food packaging isolate BC22 for meropenem and imipenem were clearly below the respective breakpoints with 13 mm and 20 mm, respectively. In addition, no single colonies were observed in the inhibition zone. Previous MIC determination via gradient test (Etest®) revealed 6 mg/L with a breakpoint 0.5 mg/L. (175) Vancomycin zone diameter for BC22, however, was 16 mm, which is far above the current breakpoint. Therefore, the findings regarding the impact of sliding motility on carbapenem and erythromycin susceptibility testing remain inconclusive.

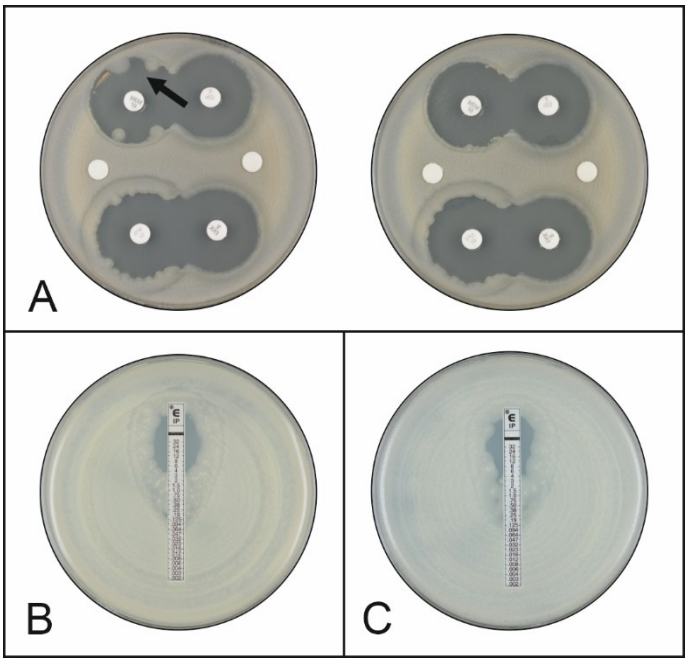

Figure 27: Ambiguous phenotypes during carbapenem susceptibility testing. A) Repeated disk diffusion testing of BC70 including MEM, CIP, LVX and IMI. Imipenem disk (bottom left) resulted in visible migration front in repeated tests. Arrow indicates sporadic single colonies protruding into inhibition zone of meropenem. B) Imipenem gradient test (Etest®) of BC70 indicated the presence of a putative migration, which resembled sliding motility. C) Imipenem gradient test (Etest®) of the vancomycin-susceptible isolate BC22 yielded a MIC of 6 mg/L (breakpoint 0.5 mg/L). (175)

Discussion

Selection of isolates for comparative studies on antimicrobial resistance

With the exception of *Bacillus anthracis*, there has been limited research conducted on *Bacillus cereus sensu lato* as a causative agent of non-gastrointestinal infections. Although many case reports have been released and summarized so far, there is only limited knowledge on the prevalence of *Bacillus cereus sensu lato* in patient samples in general. Previous studies, which were describing clinical isolates from human patients, were often limited in the number of isolates and/or focused on particular subsets of samples e.g. meningitis patients or bloodstream infections. (85,153,160,191,192) The referenced studies included 3, 17, 29, 45 and 45 isolates of *Bacillus cereus sensu lato*, respectively. This project was therefore intended to collect data on *Bacillus cereus* in a more comprehensive approach by including up to 231 patient associated isolates as well as several isolates from a related outpatient clinic environment. Furthermore, the clinical isolates were compared with environmental isolates from food packaging materials, industrial biofilms and process water. This is a common approach for antimicrobial resistance surveillance, although it has been rarely employed for the *Bacillus cereus* group. In the studies by Godič Torkar and Seme (193) as well as Godič Torkar and Bedenić (48), similar designs were chosen, as isolates from various clinical origins (wounds, burns, feces, nasal swabs etc.) were compared with food isolates in Slovenia. Nevertheless, the number of clinical isolates subjected to comparison was restricted to 30 and 29, respectively, and the reference group solely consisted of food isolates, a well-studied but not exclusive source of *Bacillus cereus sensu lato* in the environment. In general, the comparison of antimicrobial resistant bacteria from clinical samples and environmental samples is a powerful design to follow the emergence, spread and establishment of antimicrobial resistances in a bacterial population. However, selecting an appropriate environmental reference is challenging. A recent study on *Escherichia coli* compared clinical isolates from urinary tract infections with isolates from river water and aquatic biofilms in Austria. (194) Numerous other studies also compared clinical and environmental isolates, e.g., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*, typically using isolates from the hospital environment or aquatic environments as reference. (195–197) This study, however, used isolates from industrial manufacturing as comparison. Food packaging materials, including paper and cardboard, are ubiquitous in modern society and in close contact with every individual. As human-influenced environmental

samples, their characteristics as a reference are certainly unique. They are less susceptible to distinct types of confounding contamination than surface water, which can be influenced by antimicrobial resistant bacteria and their genes from wastewater and agriculture. The microorganisms present in packaging materials typically originate from the surrounding natural environment and are introduced into the production process via various means, including wood and pulp, water, dust, and air. On the other hand, they are influenced by the industrial manufacturing processes. The harsh conditions in the production process, including the use of biocides and high temperatures, may apply a certain selection pressure, which is different to natural environments. The advantage of isolates from packaging materials as reference is nevertheless the fact that it represents bacteria that are commonly surrounding us in everyday life. Given that the environment continuously shapes our microbial flora, it is similarly important to gain an understanding of the bacteria that surround us. (198) Furthermore, as this thesis was also embedded in a research collaboration with industrial partners from the packaging material sector, a definitive sample set could be obtained, thereby limiting confounding factors that would have been introduced during random environmental sampling. One potential limitation of the food packaging materials is that it may have included clonal strains, which could have affected the population under study. The origin of the food packaging isolates was described in Schmid et al. (2021) and in the master's thesis "Phylogenetic, Phenotypical and Toxigenic Characterization of *Bacillus cereus* Isolates from Packaging Materials", but included only 75 of 80 isolates. (138,175). In these studies, five isolates were previously excluded due to a possible clonal origin. The decision was based on the *panC* sequence homology. Isolates from the same packaging material showing identical *panC* sequences were suspected to be clonal. However, sequence identity of a single gene is not optimal to distinguish clonal isolates, as minor errors in Sanger sequencing can occur. While errors at the single base level, would not have affected *panC* affiliation, they could have affected the previous classification as clonal or non-clonal. Furthermore, antimicrobial resistance can also vary between individual clones, especially against the background of horizontal gene transfer and heteroresistance. (199) Heteroresistance is the phenotype in which a bacterial isolate exhibits subpopulations of cells that show significantly reduced antimicrobial susceptibility than the main population. (200) Consequently, it was decided to include all 80 isolates in the present project and subject them to AST with updated guidelines. However, although isolates with different colony morphologies were selected and differences in the *panC* gene sequence were confirmed for most of them, the presence of clonal strains cannot be ruled out. Although the isolates from 41 distinct samples,

some of the samples originated from the same production facilities and were of the same type, but not from the same production run. The same applies to the biofilm and process water samples that originated from the same production facility. Although obtained from different sampling sites, it is very likely that there is a bacterial interchange between different biofilms and the process water in one production site. Furthermore, some isolates were isolated from replicate samples, as previously indicated in the respective chapters. On top of that, a bacterial interchange between biofilm, process water and the corresponding packaging material is also probable. The potential inclusion of clonal isolates has already been identified as a risk, especially when comparing clinical isolates with environmental isolates. (194) It is challenging to achieve an appropriate balance between the need for a sufficiently large sample size and the accurate identification of bacterial clones, which requires pulsed-field gel electrophoresis (PFGE), MLST, random amplified polymorphic DNA (RAPD) PCR assays or WGS. (201) It was assumed that clonal isolates were also present in the subgroup of isolates from cranial orthoses and from the outpatient clinic, so that they were excluded from the detailed statistical analysis. Nevertheless, there is sufficient data on *Bacillus cereus sensu lato* to draw conclusions for each sample population, patient isolates and environmental isolates, and the expected bias is minimal.

Distribution of phylogenetic groups in different environments

The assignment of the isolates to their respective phylogenetic groups revealed that the diversity of *Bacillus cereus sensu lato* isolates differed according to the source from which they were obtained. A statistically significant difference was found between patient isolates from routine microbiological diagnostics and food packaging isolates. The distribution of isolates from patient diagnostics was more uniform across groups II, III, IV, V, and VI. In contrast, groups III and IV were predominant among the food packaging isolates. Although data on the phylogenetic distribution of clinical isolates is scarce, the present results are in accordance with the findings of Glasset et al. (153). The authors showed that clinical strains, which were isolated from multiple patients across France, belonged to the groups II, III and IV representing 23%, 47% and 30% of the strains, respectively. Various studies on food-borne *Bacillus cereus* s.l., showed a predominance of phylogenetic group III, accounting for 50 to 60%, coming along with a lower percentage of group II isolates. (118,202,203) A study on infant formula detected equal

frequencies of clades III and IV, and a low number of group II, (204) while a study on shellfish and aquatic products demonstrated a predominance of group IV, followed by group V. (205) In dairy-associated and food processing environments, isolates belonging to group VI prevailed, followed by group II. (174) A Japanese investigation of 44 *Bacillus cereus* s.l. isolates from various sources affiliated four patient isolates to the groups III, IV and VI, while isolates from soil and animals spread across the groups II to V. (206) In another study, 17 *Bacillus cereus* s.l. isolates from bacteremia were exclusively affiliated to groups III and IV. (192) Overall, the phylogenetic groups I and VII (*B. pseudomycoides* and *B. cytotoxicus*) appear to be extremely rare, both in clinical and environmental samples. Based on the literature, a correlation between phylogenetic distribution and a distinct source of the isolates appears to be random. Statements on the predominance of clades in a certain environment remain arbitrary, even though statistical differences can be calculated between populations. In general, studies demonstrated that a variety of clades is present in soil, air, water and plants, (31,206) and random contamination of samples, either patient-derived or environmental samples, should therefore reflect the variety in the environment. A predominance of individual phylogenetic groups can consequently be based on three causes: i) specific hygiene or contamination issues, ii) a distinct ecological niche, or iii) sampling issues. As a broad range of different phylogenetic groups was detected in the patient samples from microbiological routine diagnostics, a random, environmental origin can be assumed. Regarding the isolates obtained from food packaging materials, the more uneven distribution towards group III can be explained by the harsh environments during manufacturing. The study by Zhuang et al. (204) concluded that the heat resistance of *Bacillus cereus* spores belonging to group III was higher than that of groups IV and II. As the biofilm samples exhibited a different composition with more isolates belonging to group IV and V, a reduction of more thermosensitive isolates in the heat dried packaging materials is conceivable. In addition, a different susceptibility to biocides during the manufacturing process is possible, particularly given that biofilms typically offer enhanced protection from bactericidal effects. A bias in the random selection of isolates could be possible as not all colonies from the respective packaging materials could be investigated and some strains could have been missed out. The comprehensive examination of the phylogenetic distribution of the isolates from packaging material was published in 2021. (138)

In the set of isolates from cranial orthoses and infant’s scalps, the extreme predominance phylogenetic group III can presumably be attributed to a hygiene or contamination issue. More

than 95% of the 138 isolates from cranial orthoses and scalps of 48 different patients, and the entire outpatient clinic isolates were classified as group III, which definitely contradicts random contamination of the orthoses as suggested for the patient samples from routine diagnostics. The study, which is actually not part of this dissertation project, revealed probable cross-contamination events among patients and between patients and the outpatient clinic environment for several bacterial pathogens (unpublished results). The process of helmet fitting is likely to contribute to the spread of *Bacillus cereus sensu lato* in the outpatient clinic as viable and putatively clonal isolates were obtained from disinfecting ethanol, air, surfaces and clothes of the physician performing the fitting. The repeated sampling from cranial orthoses may have further contributed to the accumulation of clonal isolates but also supported the persistence of strains on patients. Importantly, the group III has also been considered to exhibit the highest pathogenic potential. (2) Hence, the presented results of *Bacillus cereus sensu lato* fit perfectly into picture and highlight once more the potential of the *Bacillus cereus* group as a nosocomial pathogen. It is therefore important not to limit the risk of nosocomial transmission to the classical pathogens of concern like the ESKAPE species, but to keep in mind that pathogens usually regarded as environmental species can pose a threat especially in a hospital setting. The knowledge about the antimicrobial susceptibility then provides an additional benefit for the actual risk assessment of such contamination events. However, to verify the assumption of clonal spread, typing methods with a higher resolution than *panC* gene sequencing would be required e.g., MLST or WGS. To sum up, the *panC* typing provided a sufficient overview of the isolate populations in this study. However, a meaningful conclusion about a correlation between phylogenetic groups and different environments is hardly feasible. The *panC* typing could also provide basic evidence of clonal isolates when compared with epidemiological data, thus making it a rapid and straightforward tool for preliminary classification. The previous seven-group scheme and the adjusted eight-group identification scheme yielded identical outcomes, with a single exception in the patient isolates. Nevertheless, in the context of clinical microbiology, this discrepancy is unlikely to have any significant impact. In general, both schemes provide sufficient information for phylogenetic classification and risk assessment. Recently, the genomospecies based nomenclature could be further expanded by the addition of the novel genomospecies *Bacillus rhizoplane* and *Bacillus arachidis* and the incorporation of newly published genomes into established genomospecies, proving its suitability for future taxonomic advances. (44)

Approaches for biomarkers that go beyond *panC* classification

It is also noteworthy that a correlation between distinct phylogenetic clades (e.g., *panC* groups) and the severity of non-gastrointestinal infections has yet to be proposed. Although the clinical significance in *panC* group III is assumed to be highest due to enterotoxin and potential emetic toxin and anthrax toxin production, (2) the meaning of *panC* for clinical significance is limited. The same has been concluded for sequence types based on MLST. (207) In the past, several attempts have been made to find novel biomarkers beyond the *panC* typing to differentiate between high-risk and low-risk strains within the *Bacillus cereus* group. A study aiming to identify such biomarkers used RNA-Seq to identify seven genes that were suited to predict pathogenic strains. (207) These markers were named adhB, agrC, thiJ, araC, BCQ_PI180, gshAB and BCQ_PI181 coding for an alcohol dehydrogenase, a hypothetical protein, a glutamine amidotransferase, two transcriptional regulators, a glutathione synthetase and a glutathione dehydrogenase/class III alcohol dehydrogenase, respectively. Using this set of genes, clinical strains were efficiently separated from the non-pathogenic strains, while strains associated with food-borne outbreaks and those affiliated to group IV were still challenging. Nevertheless, the authors suggested a two-step approach by combining initial *panC* typing followed by a biomarker test. (207) Another recent study sought to differentiate between gastrointestinal isolates and ocular isolates responsible for serious endophthalmitis, keratitis and blepharitis. (208) The authors compared a set of different virulence-associated genes and reported an increased prevalence of these in ocular isolates, but it had no effect on the severity of endophthalmitis in both in vitro and murine models. However, the reliability of the PCR results for the respective genes in the study is also questionable. The induction of immune cell death as a strategy of immune evasion may also be crucial in non-gastrointestinal infections. Indeed, hemolysin II (*hlyII*) has been identified in 30% of pathogenic strains associated with food poisonings and non-gastrointestinal infections, whereas it was absent in non-pathogenic strains. (209) Glasset et al. combined ten virulence genes comprising the most studied toxins of *Bacillus cereus sensu lato* and attempted to identify genetic signatures that are associated with pathogenic and non-pathogenic strains, but did not find easily interpretable conclusions for risk assessment. (210) The expression levels of the metalloproteases *inhA1* and *nprA* have also been shown to be higher in pathogenic strains than in non-pathogenic. (209) Similar results indicated a contribution of all three InhA metalloproteases (InhA1, InhA2, InhA3) to the severity of infection and inflammation in endophthalmitis. (211) The supply of virulence factors in *Bacillus*

cereus sensu lato is nevertheless inexhaustible, ranging from well-studied (entero-) toxins to degradative enzymes and multicellular behavior. As a consequence, it seems impractical to attribute pathogenic potential to individual virulence factors or even their expression. The discovery of reliable biomarkers for high-pathogenic strains and individual virulence genes (*ces*, *hbl*, *nhe*, *cytK*) would likely offer significant benefits in epidemiological research, particularly in the identification of risk environments, such as specific food products or hospitals. The fact that the *Bacillus cereus* group is also part of the transient microbial flora of the gut underscores the need to differentiate pathogenic from non-pathogenic, if possible. (212) However, the implementation of sophisticated methodologies, such as gene expression analyses or the identification of multiple virulence genes are not feasible on a routine basis. In a clinical context, a straightforward assay beyond the phylogenetic *panC* classification to assess the pathogenic potential could also enhance the diagnosis, interpretation, and eventual treatment of non-gastrointestinal infections.

Reporting *Bacillus cereus sensu lato* in a clinical laboratory

The data indicated that phylogenetic groups are not particularly associated with either human patients in general or the subset of positive blood cultures. The risk of infecting a patient, colonizing a patient, or contaminating a patient sample was hence not restricted to any phylogenetic clade. An outstanding feature of this project was the inclusion of all *Bacillus cereus sensu lato* isolates present in patient samples, taking also into account putative sample contaminations or isolates that might not have contributed to the underlying disease. The advantage of this strategy was to depict the actual variety of *Bacillus cereus* species and strains associated with all kinds of patients and to link this information with the antimicrobial susceptibility. Endogenous transmission of pathogens from the human bacterial flora is one of the main causes of infections, especially healthcare-associated infections. (213–215) It is therefore important to shine also a light on neglected members of the endogenous flora for a proper risk assessment. Moreover, the stigma of *Bacillus cereus* as a frequent sample contamination is likely to result in reduced reporting and, consequently, restricted AST. A true retrospective quantification of *Bacillus cereus sensu lato* in patient samples is further complicated by varying reporting practice of clinical microbiologists. In clinical samples with suspected contamination, *Bacillus cereus* may be specified solely as *Bacillus*, aerobic spore

former, gram-positive rod, or not at all. The present study could also not fully elucidate the total prevalence of *Bacillus cereus sensu lato* in patient samples from microbiological diagnostics and an accurate quantification was not feasible due to varying reporting and identification frequencies by biomedical scientists and clinical microbiologists. In this project, the actual relevance of *Bacillus cereus sensu lato* in the respective patient sample was not resolved. The evaluation of how the *Bacillus cereus* group was reported in the final microbiological findings revealed that individual interpretation of the sample influenced the reporting manner. For severe cases (e.g. blood cultures, infected burns), a higher level of information was provided, and AST was always included with the full species designation in the microbiological report. In several other cases, *Bacillus cereus sensu lato* was outlined as aerobic spore former or *Bacillus* spp. However, a species identification must have already been performed. Without this identification, the isolates would not have been provided for this study. The reason for this reduction in information in the microbiological report remains questionable. Although clinical interpretation of the microbiological reports was not done systemically, individual cases can be subject for debate. One isolate was obtained from a patient with acute eyelid swelling and suspected blepharitis, but AST was not provided. A similar microbiological report was made in cases of periorbital dermatitis at the canthus and eczema at the upper eyelid. The latter was co-infected with *Corynebacterium macginleyi*. Since the *Bacillus cereus* group is among the most devastating etiological agents of serious (intraocular) eye infections, AST could have been a benefit. (50) The patient samples also comprised several fecal samples of acute gastroenteritis, which were screened for diarrheal pathogens. Although the *Bacillus cereus* group has been identified and provided for the study, it was never mentioned in the findings. In these cases, the microbiological reports only referenced the presence of serious enteropathogens (*Salmonella* sp., *Shigella* sp. *Campylobacter coli*/*jejuni*, *Yersinia enterocolitica,* enterohemorrhagic *Escherichia coli*), which are known for causing dreaded gastroenteritis and are obliged to registration by Austrian federal law. In fact, none of these were detected in the respective samples, while *Bacillus cereus sensu lato* is a notorious agent of gastroenteritis. However, it is also present in the transient microbial flora of the gut, which may contribute to the underreporting of cases. This is an illustrative example of the reasons why the number of unreported cases of *Bacillus cereus* gastroenteritis is estimated to be considerably higher. (216)

One isolate from microbiological patient diagnostics was retrospectively identified as collected from a cranial orthosis. Since it was not part of the clinical trial on helmet therapy, it was not

included in the population of isolates from helmet orthoses. Moreover, the fact that the sample was sent to the Department of Bacteriology indicates that it originated from a genuine infection. The microbiological report also included AST results. In many cases, the detection of the *Bacillus cereus* group indeed had no relevance such as in MRSA screening via nasal swabs. It is very reasonable not to over-report microbiological results. Conversely, the role and contribution of *Bacillus cereus sensu lato* in samples comprising a diverse bacterial community including apparent pathogens is a legitimate subject for debate. It is unclear whether a high amount of *Bacillus cereus sensu lato* in a case of balanitis is a relevant factor. Does *Bacillus cereus sensu lato* contribute to the development of pharyngitis when present in massive quantities? Is it favorable to provide AST for *Bacillus cereus sensu lato* when detected in significant numbers in wound swabs together with *Staphylococcus aureus* or *Klebsiella pneumonia*? Overall, the accurate interpretation of *Bacillus cereus* in clinical samples is still challenging, starting with the manner of reporting up to the decision of AST or not. To address the current knowledge gap, further studies are required to ascertain the actual prevalence of *Bacillus cereus* in patient samples.

Antimicrobial susceptibility (testing) of *Bacillus cereus sensu lato*

The antimicrobial susceptibility of the *Bacillus cereus* group has received only marginal attention in the past, which is well reflected in the late release of breakpoints and zone diameters by EUCAST and differing breakpoints in the CLSI and EUCAST guidelines. (171,177,217) Some of the patient isolates from microbiological routine diagnostics, as well as the food packaging isolates have previously been tested for their antimicrobial susceptibility, either using gradient tests or the disk diffusion method with adopted breakpoints. All isolates were therefore re-tested in accordance with the current EUCAST breakpoints for *Bacillus* spp. In the EUCAST guidelines, norfloxacin is recommended as a surrogate agent for epidemiological screening for fluoroquinolone resistance in *Bacillus* spp. However, since ciprofloxacin and levofloxacin are indeed clinically relevant antimicrobials, these agents were tested directly. The conclusion that patient isolates comprised more resistances to relevant antimicrobials was based on two findings: i) a statistically significant difference in the distribution of resistant phenotypes and ii) higher proportions of isolates exhibiting one resistance and three or more resistances. This observation is in accordance with other comparisons of clinical and environmental isolates,

which concluded that antimicrobial resistances are more frequent in patient isolates. (194–196) In general, the increased proportion of antimicrobial resistances in patient isolates can be attributed to the use of antibiotics in the treatment of infections and the concomitant selective pressure for resistant bacteria to survive. Moreover, the prevalence of antimicrobial resistant bacteria is much higher in healthcare institutions and the risk for transmission is therefore elevated. In patients, bacteria also find conditions that favor horizontal gene transfer, while natural environments with diverse ecosystems usually counteract the spread of antimicrobial resistance genes. (218) Nonetheless, antimicrobial resistances are likely to be ubiquitous in the *Bacillus cereus* group as evidenced by the reported antimicrobial resistances among the isolates from food packaging materials. However, food packaging materials do not provide a reservoir of particularly resistant strains. In 2019, Fiedler et al. (114) reported resistances to erythromycin, ciprofloxacin, gentamicin or imipenem in a set *Bacillus cereus sensu lato* from fresh vegetables in Germany. The authors initially propagated minimal resistance rates of 4.1%, 0.7% and 0.7% to erythromycin, ciprofloxacin and imipenem, respectively. However, they applied EUCAST breakpoints from *Staphylococcus* and *Enterococcus*, which were substantially different from current breakpoints for *Bacillus* spp. A re-evaluation of the results from the food packaging isolates of this dissertation project with the expired breakpoints from Staphylococcus and Enterococcus as used by Fiedler et al. (114) would yield even lower resistance rates of 1.3%, 0.0%, and 0.0% for erythromycin, ciprofloxacin, and imipenem, respectively. Similarly, low rates of antimicrobial resistant isolates were also reported for dairy products and dairy-associated environments, ice cream, vegetables, leaves, seeds, water and soil. (174,202,219,220) A study on 95 strains from various natural environments and strain collections also revealed resistances to clindamycin, erythromycin and meropenem using gradient tests and broth microdilution, but they also applied CLSI breakpoints for *Staphylococcus aureus*. (221) A study on ready-to-eat foods in China also used CLSI breakpoints for *Staphylococcus aureus* and reported 2.72% erythromycin-resistant isolates but more than 50% intermediate isolates, which would all be classified as resistant when applying current breakpoints by EUCAST. (122) Current breakpoints would also result in more than 20% ciprofloxacin resistant isolates. Moreover, they reported significant numbers of isolates exhibiting resistances to clindamycin or tetracycline. Nakayama et al. (222) identified 100% tetracycline and ciprofloxacin resistance among 12 isolates from retail chicken when applying *Staphylococcus aureus* breakpoints by EUCAST. Overall, the heterogeneity in applied breakpoints hampers the comparability of individual studies. Moreover, the fact that many studies still assess beta-lactam susceptibility despite well-known intrinsic

resistance throws doubt on the scientific rigor. A shift to the currently valid breakpoints by EUCAST would increase the number of environmental *Bacillus cereus* group isolates that are classified as resistant to a clinically relevant antimicrobial agent.

The presence of various antimicrobial-resistant strains in natural and human-associated environments, along with their statistically significant but relatively modest prevalence among patient isolates, suggests that the strains colonizing and infecting patients originate from the environment. This includes sources such as food, soil, dust, air, and water. Godič Torkar and Bedenić (48) also suggested an environmental source of clinical isolates, as they could not find significant differences in MIC values between environmental and clinical *Bacillus cereus* group isolates. Based on the results of this dissertation, strains with increased resistance accumulate in patients and the healthcare setting in general. Although the difference between patient isolates and environmental isolates was not striking, the results confirm that the accumulation of antimicrobial resistance in clinically relevant settings is not limited to the species of concern that clinical microbiologists are aware of (e.g. ESKAPE bacteria) but also expand to hitherto neglected pathogens.

Although the frequencies of resistant phenotypes significantly differed between the patient isolates and environmental isolates, no post-hoc tests were carried out to determine whether there is a statistically significant difference in the number of multi-drug-resistant isolates. Antimicrobial resistances are not necessarily independent from each other and can have the same underlying mechanism. Resistance to one antimicrobial may influence the susceptibility to another, which would introduce a bias in the statistical analysis. Examples are a combined resistance to macrolides, lincosamides and streptogramin B due to target methylation, or a resistance to fluoroquinolones affecting both ciprofloxacin and levofloxacin due to efflux pumps. (223,224) One potential solution to this issue is to consolidate the individual agents into classes and then perform a comparison. However, no evidence of MLS resistance, as indicated by the presence of inducible clindamycin resistance (the D phenomenon), (223) and only one concomitant resistance to ciprofloxacin and levofloxacin were observed in the course of this project. Therefore, a comparison of classes was not performed. The different frequencies of antimicrobial resistant phenotypes may also be affected by the differing phylogenetic distribution between patient isolates and environmental isolates. However, no particular attention was paid to possible correlations and a previous study did not suggest a relation. (174)

The samples from patient diagnostics comprised the highest rates of isolates exhibiting resistance to clindamycin (3.23%), erythromycin (33.33%), vancomycin (10.75%) and imipenem (23.66%) in disk diffusion testing. A comprehensive review by Lotte et al. (147) summarized clinical data on the treatment of *Bacillus cereus sensu lato* infections and reported resistance rates of 26%, 25%, 5% and 13% for clindamycin, erythromycin, vancomycin and imipenem, respectively. Again, re-evaluating the data with different, outdated breakpoints for *Staphylococcus* and *Enterococcus* would markedly alter the documented resistance rates to 69.89%, 23.66%, 21.51%, and 3.23% for the aforementioned antimicrobials. A high susceptibility to linezolid and fluoroquinolones could be confirmed in this project. The well-summarized conclusions on resistance rates by Lotte et al. (147) included findings from numerous studies applying different methods and breakpoints. Therefore, they are probably only comparable to a limited extent and are not suitable for solid epidemiological interpretation. Two studies specifically dealt with the antimicrobial susceptibility of more than twenty and seventeen *Bacillus cereus* group isolates from human bacteremia, respectively. Ikeda et al. (160) and reported 65.5%, 37.9%, and 10.3% of the isolates being resistant or intermediate to clindamycin, erythromycin, and levofloxacin, respectively. On the contrary, all isolates were reported as susceptible to vancomycin, imipenem and linezolid. In the study by Bianco et al. (192), one isolate was resistant to clindamycin, while four isolates demonstrated intermediate resistance to clindamycin and/or erythromycin. All isolates were susceptible to vancomycin, linezolid and ciprofloxacin. Both studies used the broth microdilution method for AST. In this dissertation project, the limited number of isolates obtained from positive blood cultures (n = 8) impedes a meaningful comparison of antimicrobial susceptibility. However, the high rate of erythromycin resistance and occasional clindamycin resistance, as well as 100% susceptibility to linezolid and fluoroquinolones is in line with the other studies. The reduced prevalence of antimicrobial resistances among the isolates from cranial orthoses is probably due to clonal isolates, as discussed before.

The molecular fundamentals for antimicrobial resistances apart from beta-lactam resistance are mostly unclear for the *Bacillus cereus* group. In this project, the interference of a motility strategy with accurate vancomycin susceptibility testing could be described for the first time. This finding has relevant implications for susceptibility testing, as vancomycin is considered a drug-of-choice for empirical therapy of severe non-gastrointestinal infections with *Bacillus cereus sensu lato*. (147,225,226) Numerous publications have reported a 100% susceptibility of *B. cereus* s.l.

isolates to vancomycin, (153,160,192,227,228) whereas true vancomycin resistance is rather a sporadic exception. Of particular interest is the observation that vancomycin-resistant isolates were frequently identified through the use of the disk diffusion method. (229–231) This method is widely used for antimicrobial susceptibility testing due to its fast and straightforward application. The release of standardized zone diameters by EUCAST in 2021 will probably further contribute to the propagation of disk diffusion, as it is no longer necessary to fall back on *Staphylococcus* spp. zone diameters from the CLSI M100 guidelines. Although definitive zone diameters are now available for *Bacillus cereus sensu lato*, the results of this project indicate that current guidelines are not satisfactory for the interpretation of vancomycin susceptibility testing. The recent study by Mills et al. (174) supports these findings, as they concluded that disk diffusion testing is not sufficient in the evaluation of antimicrobial susceptibility in the *Bacillus cereus* group. Despite a high antimicrobial susceptibility, there was only weak correlation between zone diameters and the MIC, which was determined via broth microdilution. The authors therefore advised to avoid disk diffusion for AST. It is noteworthy that none of the 85 isolates from food and dairy processing environments included in the study by Mills et al. (174) was reported as resistant to vancomycin in disk diffusion testing. However, two isolates were resistant in broth microdilution and were concomitantly resistant to linezolid. The MIC was above 128 mg/L and the isolates were assigned to the phylogenetic groups II and V. In the study by Godič Torkar and Bedenić (48), a single isolate was resistant to vancomycin, exhibiting a MIC of 16 mg/L in broth microdilution. Moreover, three isolates were reported as intermediate, a classification that is no longer in use by EUCAST. In fact, the authors fell back on interpretative standards of MICs for *Staphylococcus* spp. by CLSI, defining a breakpoint of 2 mg/L and a range of 4 to 8 mg/L for classifying intermediate. It highlights again the difficulty in accurate interpretation of AST for the *Bacillus cereus* group, resulting from the broad spectrum of current and outdated interpretive criteria in the literature.

A significant limitation in medical microbiology is that putative resistance in disk diffusion is frequently corroborated by the gradient test method (Etest®), which was shown to yield identical outcomes to those observed in disk diffusion. The reference methods broth microdilution and agar dilution, however, are rarely used in routine microbiology laboratory settings due to their required labor input and high costs. The findings of this project, however, indicate that only phenotypical methods, which are independent from agar diffusion, should be used to obtain reliable results regarding vancomycin susceptibility.

The evaluation of vancomycin resistance with reference methods also revealed trailing growth and putative heteroresistance as phenomena in the *Bacillus cereus* group. Trailing endpoints were occasionally observed for agar dilution. In several cases, growth did not occur in individual replicate wells when broth microdilution was employed. This involved various concentrations below the MIC. In addition, there was a consistent discrepancy of one dilution step between broth microdilution and agar dilution. Due to successful quality control for the inoculum and correct matches of the MIC for the reference strain, this may be due to a random error. Inter- and intra-laboratory variability in AST and MIC determination is a well-known observation, despite good reproducibility overall. (232) A range of one dilution step is also acceptable for EUCAST quality control and within the expected reproducibility. (180,181) For vancomycin however, it is known that there is poor reproducibility between different methods of AST and the value of reporting a MIC has been up for debate. (233,234) Furthermore, broad prevalence of heteroresistance to carbapenems has been identified among *Bacillus cereus* isolates, a phenomenon that has already been described for other species including *Pseudomonas aeruginosa, Enterobacter cloacae* and *Salmonella enterica*. (200)

Transcriptome analysis and sliding motility

This study also aimed to understand the cellular mechanisms responsible for the misinterpretation of vancomycin agar diffusion testing in the *Bacillus cereus* group. While a fixed concentration of vancomycin above the MIC consistently prevented growth, methods that rely on a vancomycin gradient in solid media, such as disk diffusion or gradient tests, resulted in growth beyond the actual MIC. Based on the phenotypical experiments on the prima facie highly resistant food packaging isolate BC70, a motility driven expansion of *Bacillus cereus* s.l. on the agar surface was discovered. A variety of strategies have been described in bacteria for translocation within liquids or on surfaces. (235) Swarming motility is a strategy of various bacteria to actively move on surfaces and it should be cautiously handled when interpreting inhibition zones. (176,236) Although its ability to swarming motility has been shown, (47) *Bacillus cereus* s.l. has not yet been recognized as a swarming species that could interfere with the agar diffusion test. In contrast to swarming motility, this dissertation project revealed that sliding motility is the underlying mechanism responsible for the observed translocation across the agar surface into the inhibition zone. Bacterial sliding is a passive mechanism that is mainly driven by

the expansive forces of cell division and the synthesis of friction reducing components e.g. surfactants. (237) The molecular machinery involved in sliding motility may overlap with the machinery required for biofilm formation and is not dependent on the flagellar system. Both, phenotypical and gene expression assays led to this conclusion. During surface translocation of BC70, a gene expression pattern that is typically associated with biofilm cells was identified. (179) The GO analysis revealed pronounced differences in the extracellular region of BC70 compared to ATCC 14579, which was reflected in the increased expression of *calY* and *tasA*, encoding two biofilm matrix proteins that form structural fibrils in the extracellular matrix. (89,90,238) Together with the signal peptidase SipW, which is required for their activation, CalY and TasA have been shown to contribute to biofilm formation by building an amyloid-like fiber network. Candela et al. (90) reported that *calY* was by far the most overexpressed gene in *Bacillus thuringiensis* biofilms, a result which could be confirmed with this study. Furthermore, CalY has been associated with adhesion to epithelial cells, making it a putative virulence factor. The homolog in *Bacillus subtilis*, *tasA*, is essential for proper colony expansion and is likely to be involved in fine-tuning the formation of van Gogh bundles. (186) During bacterial sliding, TasA specifically localizes to the pole-to-pole interaction zones of TasA-producing cells inside the van Gogh bundle and may affect the folding properties and thereby the efficiency of migration.

The lipopeptide kurstakin, encoded by the *krs* locus, is involved in proper biofilm structuration. (239,240) Kurstakins were described as heptapeptides (Thr-Gly-Ala-Ser-His-Gln-Gln) with a varying fatty acid residue. (241) In general, lipopeptides are known for their surfactant properties and therefore play a major role in reducing surface tension for bacterial motility and biofilm formation. (242) Similar to surfactin in *Bacillus subtilis*, kurstakin may function as the surfactant that is essential for sliding motility. (186,243,244) The role of kurtstakin in multicellular motility has also been described by Yu et al. (239) in an assay for swarming motility. However, based on the observations during this project, it can be challenging to establish a true swarming population of *Bacillus cereus*, even when low concentrations of agar are utilized. Thus, it is possible that the referenced study actually monitored sliding motility rather than swarming motility.

As in other studies on *Bacillus cereus sensu lato* biofilms, not all transcripts of actually co-expressed genes could be detected, which demonstrates how variable gene expression studies on biofilms and motility still are. (190,245,246) For example, the comparison of cells from gradient test plates between BC70 and ATCC 14579 revealed three differentially expressed

genes of the *hblDAC(B)* operon, but the comparison between BC70 cells from the gradient test plate with swarming cells revealed four of them. The operon exists with three or four open reading frames due to a presumed gene duplication. The *Bacillus cereus* reference strain (ATCC 14579) possesses the four gene *hblDAC(B)* operon variant. Enhanced transcription of *eps* operons, which are related to the production extracellular polysaccharides and necessary for sliding motility and van Gogh bundles, (186,243) was not detected. Additionally, the *sipW-tasA* bicistronic operon should be co-expressed, but only one or the other has been detected in this study. (179)

Increased antimicrobial resistance is a common characteristic of biofilms and has also been demonstrated for swarming cells due to high cell densities paired with mobility. (247) This may act synergistically with a putative barrier function of a surfactant to facilitate the survival of BC70 upon vancomycin exposure. Since pseudo-resistance was mainly observed with vancomycin, the questions of whether the specific effect of vancomycin on peptidoglycan synthesis plays a role or whether the cells are shielded from vancomycin by biofilm matrix or surfactant, remain unanswered. (179) A 4-fold higher MIC of daptomycin was also observed for swarming cells of *Bacillus cereus* ATCC 14579 in comparison to non-motile cells. (183) However, the increased resistance was not attributed to higher cell densities, but to the increased expression of previously unnoticed resistance-mediating genes under swarming conditions. These results indicate that the expression of unheeded genes may also be influenced by multicellular behavior and thereby affect a (pseudo-)resistant phenotype.

The transcriptomic analysis confirmed once more that biofilm and sporulation pathways are intertwined in *Bacillus cereus sensu lato*. The increased expression of the regulator encoding genes *spoVG* and *comER*, which both contribute to biofilm formation and sporulation, (188,189) further corroborates the involvement of the biofilm machinery and thereby the sliding motility. SpoVG has been shown to regulate the transcription levels of Spo0A, which in turn governs sliding motility in *Bacillus subtilis*. (243) In fact, Grau et al. (243) showed a sequential transition from sliding motility to sessile biofilm and sporulation explaining the upregulation of genes related to sporulation in this study. (179) From an ecological perspective, this transition facilitates the *Bacillus cereus* group to conquer new natural environments by translocation, establishing a robust colony in a biofilm and turn into resistant spores to endure periods of harsh conditions until re-germination. Actually, sliding motility accompanied by van Gogh bundles has been described in *Bacillus cereus* under nutrient starvation. (244) However, this ecological

perspective could also be transposed in a clinical context, when *Bacillus cereus* enters a host e.g., via surgery or a catheter, followed by dissemination on a surface like implants, prostheses or catheters, and the formation of biofilm to resist antimicrobial treatment. Then, the eventual formation of endospores could further hamper the elimination of *Bacillus cereus* and prolong the infection. This threat of biofilms by *Bacillus cereus sensu lato* has indeed been spotlighted by Gurler et al. (92), who concluded that slime production makes *Bacillus cereus* cells highly adherent to the catheters. The increased resistance within catheter-associated biofilms results in an unsatisfactory clinical outcome due to inactivity of vancomycin against *Bacillus cereus sensu lato* dwelling in the biofilm layer.

Similar to nutrient starvation, other stressors such as antibiotic exposure could facilitate a sliding phenotype. The sliding motility of *B. subtilis* was indeed activated upon exposure to subinhibitory concentrations of several antibiotics, but vancomycin was not tested. (248) In addition, sliding motility was also suspected in small colony variants (SCV) of *Bacillus cereus* induced upon exposure to aminoglycosides. (179) The SCV formed atypically smaller colonies with slower growth behavior. Microscopic examination revealed that cells of SCV were arranged in long chains with failed cell separation. (249) Moreover, SCV also showed strongly reduced beta-hemolysis, which is consistent with the repressed expression of hemolytic toxins in this project. The reduced expression of *hblDAC(B), nheABC*, and other virulence factors relies on the repression of the PlcR virulence regulator via Spo0A and the sporulation/biofilm pathway. (250) A reduced or absent hemolysis due to a sliding phenotype may have further implications for the identification of *Bacillus cereus* s.l., as traditional protocols may still rely on hemolysis to differentiate *Bacillus cereus* from *Bacillus anthracis*. In fact, the non-hemolytic phenotype in *Bacillus anthracis* and some other strains of the *Bacillus cereus* group are based on a mutation in the *plcR* gene. (251)

Even clinical microbiologists are mostly unfamiliar with sliding motility compared to the well-studied swarming motility. Consequently, experienced clinical microbiologists involved in patient diagnostics initially classified the observed spreading phenotype of BC70 as swarming motility. In the course of this study, swarming motility was eventually refuted not only due to the absence of hyperflagellated cells, but also due to increased expression of *mogR*. The transcriptional regulator MogR (motility gene repressor) is a repressor of flagellar motility and virulence while promoting biofilm-associated gene expression. (187) In addition, swarming motility is

accompanied by an elevated expression of virulence genes that are regulated by the transcription factor PlcR. (183)

Other manifestations of multicellular behavior in the *Bacillus cereus* group

The findings of this project contribute to the growing body of knowledge regarding multicellular behavior in the *Bacillus cereus* group. Indeed, the filamentous bacteria that have long been known to inhabit the guts of arthropods, and probably other species as well, can be attributed to the *Bacillus cereus* group. (252) Moreover, it has been shown that *Bacillus cereus sensu lato* uses a multicellular motility mechanism for active soil translocation, which is independent from flagella-driven swarming. (253) In *Bacillus thuringiensis,* a similar phenotype with alternative surface translocation and translucent colonies has recently been termed the “fuzzy spreader” morphotype. (254) It has been described as a biofilm-specialist with enhanced biofilm productivity and translocation. However, whether this morphotype is connected to sliding motility remains still unclear. Caro-Astorga et al. (255) identified the exopolysaccharide locus *eps1*, but not the biofilm-associated *eps2* locus, as relevant for a kind of social bacterial motility in the *Bacillus cereus* group and thereby substantiating the results on sliding motility. (186) Another recent description of filamentous motility in a *Bacillus cereus* s.l. strain also revealed a so far unknown type of motility. (256) While the microscopic images showed similar chains of elongated cells, the rhizoid growth and gene expression profiles differed significantly. The authors reported an increased expression of inosine monophosphate and virulence genes with concomitant repression of sporulation genes. Overall, there is a great diversity of mechanisms for bacterial movement that have yet to be fully elucidated. The implications of these mechanisms in other contexts, such as infection biology, remain to be investigated further. Future research should therefore concentrate on possible effects of sliding motility in *Bacillus cereus* infections. Sliding motility and biofilms may provide increased resistance to antimicrobial therapy. On the contrary, this phenotype appears to be less virulent in terms of toxin expression and other key virulence factors. In vivo experiments may therefore contribute to understanding its role in a clinical context. In addition, the genetic background of this sliding phenotype should be addressed in detail, e.g., through WGS to identify the unique regulatory features. (179)

Conclusions and unresolved issues

To sum up, pseudo-resistance to vancomycin was reported for 18 out of 353 isolates. The disproved vancomycin resistance has a minimal impact on the data on antimicrobial resistance and its interpretation in patient and environmental isolates, as it occurred with a similar frequency of 10% and 7.5%, respectively. The mechanism of sliding motility to mimic vancomycin resistance, however, was only investigated in the BC70 isolate. Nevertheless, is very likely to be the responsible mechanisms in the remaining isolates and it could be more prevalent than previously noted. In a recent study by Baghbadorani et al., (257) more than 20% of *Bacillus cereus* isolates from spices were reported as vancomycin-resistant via disk diffusion testing. Future research should therefore broaden the knowledge on sliding motility in the *Bacillus cereus* group and its interference with susceptibility testing of other antimicrobials. Based on the results, sliding motility might also affect possible carbapenem-resistant phenotypes. A migration front similar to bacterial sliding or swarming motility was observed for numerous isolates at various extent. The large zone diameter of carbapenems allowed a better differentiation of initial inhibition zones and subsequent translocation even in disk diffusion tests, which was not discernible for vancomycin disks. As a result, a putatively motility-driven pseudo-resistance is easier to detect and likely to be assumed. However, there was no consistency in vancomycin pseudo-resistance and carbapenem-resistant phenotypes. Experienced clinical microbiologists initially classified the migration front in carbapenem disk diffusion as swarming motility, which should not be considered in AST. For this reason, the BC70 isolate was initially reported as susceptible to imipenem based on interpretations of clinical microbiologists. (179) Since the mere zone diameter for imipenem is below the breakpoint, the isolate is nevertheless listed as resistant in this dissertation. In the absence of specific guidelines, both interpretations - resistant and susceptible – may be valid. Overall, there are still many blind spots and challenges to overcome in testing the antimicrobial susceptibility of the *Bacillus cereus* group. Up to now, the culture-based approach to antimicrobial susceptibility testing has no real alternative, as the mere detection of genes presumed to be associated with vancomycin resistance has never been consistent with phenotypic resistance. (174,192,202) This dissertation corroborates the recent conclusion put forth by Mills et al. (174) that agar diffusion tests are often inadequate for the *Bacillus cereus* group. Nevertheless, as long as pertinent guidelines fail to address this issue, agar diffusion tests will be utilized for microbiological diagnostics. Therefore, the presented results can be a point of reference for what to expect and where to take a closer look.

Although there is no evidence yet of a resistance problem in the *Bacillus cereus* group, there are (probably acquired) resistances present in both clinical and environmental isolates. For vancomycin susceptibility testing, sliding motility should be implemented in current guidelines on AST interpretation to ensure reliable susceptibility testing and maintain important therapeutic options for the treatment of severe *Bacillus cereus* infections. (179)

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