CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Identification of Genomic Diversity in *Acinetobacter baumannii* Genome via Pangenome Analysis

by

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I dedicated those theses to all the great people came in my life specially my beloved parents and my supervisor who encouraged me to stand out in this world with nobility and motivated me to step ahead without any fear

CERTIFICATE OF APPROVAL

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(Zoha Ghous)

Abstract

Acinetobacter baumannii, classified as a gram-negative coccobacillus lacking flagella, is a major cause of nosocomial infections, affecting various body systems in all age groups, especially neonates. Despite its significance, genetic diversity studies in specific regions, like Pakistan, are limited. This research explores *A. baumannii* strains' genetic composition through genome sequencing and analysis from local sources. Comprehensive techniques, including sequencing, assembly, and annotation, are employed to elucidate genomic architecture. A dataset of 196 genomes, including 28 Pakistani drafts, is analyzed, revealing strain BAC8924 belonging to ST2. MLST analysis using seven housekeeping genes indicates genetic diversity. Pangenome analysis of 216 *A. baumannii* genomes identifies 19,001 genes, with core, soft core, accessory, and unique genes distributed as 1,326, 512, 2,695, and 25,894, respectively. The ST2-based pangenome analysis shows 1407 genes, including 799 soft core, 1461 accessory, and 9773 unique genes. Core-genome phylogenetic analysis highlights clades among global strains, including BAC8924. SNP-based phylogeny within ST2 reveals subclades, such as BAC8924, Pesh13, Pesh11, and Pesh06. This study unravels the genetic diversity of *A. baumannii* strains in Pakistan, shedding light on their genomic architecture. The identification of distinct subclades within ST2, exemplified by BAC8924, contributes to our understanding of *A. baumannii* evolution. Future research should explore the functional significance of unique genes and assess the clinical implications of genetic variations. Additionally, ongoing surveillance and genomic studies are crucial for monitoring *A. baumannii*'s evolution and devising effective strategies to combat nosocomial infections.

Keywords: *Acinetobacter baumannii*, nosocomial infections, Multilocus Sequences Typing, Pangenome Analysis

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Abbreviations

Bap	Biofilm-associated proteins
CDC	Centers for Disease Control and Prevention
COGs	Clusters of Orthologous Groups
CGE	Center of Genomic Epidemiology
ESKAPE	Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa and Enterobacter
HAP	Hospital Acquired Pneumonia
ICU	Intensive Care Units
ITOL	Interactive Tree of Life
LPS	Lipopolysaccharide
MLST	Multi-Locus Sequence Typing
OMVs	Outer Membrane Vesicles
PubMLST	Public Multilocus Sequence Typing
RAST	Rapid Annotation Utilizing Subsystem Technology
UTIs	Outer Membrane Proteins
VAP	Ventilator Associated Pneumonia
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background

Gram-negative bacilli that are both normal and pathogenic are grouped together in the genus Acinetobacter. *Acinetobacter baumannii*, one of the species in this genus, has gained notoriety as a significant, widely spread hospital-acquired pathogen. Numerous nosocomial diseases, including meningitis, sepsis, urinary tract infections, wound and burn infections, and pneumonia, are caused by this species. The number of strains of *A. baumannii* that resist several drugs is rising, and many strains are resistant to all antibiotics that are relevant to clinical practice. Numerous strains of *A. baumannii* have emerged as a result of the species' fast development; nevertheless, two widely dispersed Global Clone I (IC1) and Global Clone II (IC2) are two clonal lineages now dominate the species (1).

Clinical management of individuals infected with *A. baumannii* becomes more challenging due to the rising number of isolates resistant to antibiotics. Particularly among critically ill patients multidrug-resistant (MDR) in intensive care units (ICUs) *A. baumannii* strains are mostly to blame for infections linked to ventilator-associated pneumonia, infections of the bloodstream, infections of the urinary tract, and skin and soft tissue infections. Healthcare-associated isolates exhibit a significant degree of variability, as shown by phenotypic and genotypic

investigations (2). The relationships between the *A. baumannii* isolates were investigated using multilocus sequence typing (MLST) approaches (3). Housekeeping gene sequences (cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB) can be compared, this typing technique makes it easier to distinguish between microbiological isolates and enables research into different sequence types' (STs) dispersion and spread. When it became apparent that unique bacterial lineages occurred and propagated in accordance with a particular geographic distribution, several *A. baumannii* international clones (ICs) were classified according to continents. Consequently, because of their exceptional genomic plasticity—which makes them Anthropogenic *A. baumannii* isolates dispersed internationally demonstrated local clonal proliferation, making them appropriate for obtaining and/or upregulating foreign genes to swiftly adapt to environmental/host changes (4). Through the use of genome sequencing techniques, we were able to identify resistance genes and genetic components unique to particular isolates of *A. baumannii*. To ensure that comprehend the aim of this work is to analyze the genomes of isolated strains of *A. baumannii* and compare them with other sequences of *A. baumannii* pathogenesis that are currently accessible, taking into account the adaptive changes that each strain undergoes. Numerous species, such as bacteria, fungi, viruses, parasites, and other agents, can cause nosocomial infections. Exogenous or endogenous sources can give rise to infections, which can be spread by direct or indirect contact between patients, healthcare providers, contaminated objects, visitors, or even different environmental sources (5)

Antimicrobial-resistant bacteria are becoming more prevalent and are linked to nosocomial infections. This puts a heavy strain on healthcare systems and has substantial financial implications for the entire world. High rates of death and morbidity, higher treatment expenses, uncertainty surrounding diagnosis, and mistrust of conventional medicine are some of the effects. A set of nosocomial pathogens has started to be referred to as "ESKAPE pathogens" in recent research that use information gathered from hospital-based surveillance studies and the Infectious Diseases Society of America (6).

The collection(5) of bacteria known as ESKAPE is made up of Enterococcus

faecium, Staphylococcus aureus, Klebsiella pneumoniae, *Acinetobacter baumannii*, Pseudomonas aeruginosa, and Enterobacter species. Both Gram-positive and Gram-negative organisms are included. These bacteria, which are common causes of potentially fatal nosocomial infections in critically ill and immunocompromised individuals, describe potential treatment resistance mechanisms (7). Risks should be raised about *A. baumannii*'s the ability to create biofilms and exhibit resistance to desiccation and cleaning agents, as these characteristics to ensure its effective persistence and spread in healthcare environments. Features of *A. baumannii*'s DNA include environmental persistence, antibiotic resistance, and the absence of recognized host-damaging toxins, indicating that the pathogenicity potential of this organism is probably due to a strategy based on persistence and resistance. These bacterial species are also resistant to oxidative stress and complement-mediated death. Because of the significant genetic variation among isolates caused by *A. baumannii*'s malleable genome, studying *A. baumannii* as a distinct organism is made more difficult (8). Therefore, the public health sector must maintain constant surveillance due to the ongoing global issue posed by the prevalence of *A. baumannii.* The microorganism exhibits rapid growth on commonly used laboratory culture medium, such as CHROMagar, Blood agar, and MacConkey agar. Following an incubation period lasting between 18 and 24 hours at a temperature of 37°C, the organism demonstrates the emergence of smooth, glossy, and mucoid colonies on blood agar. The observed colonies exhibit a lack of color, do not cause hemolysis, and possess a diameter that falls within the range of 1 to 2 millimeters. The bacterium demonstrates the attribute of generating colorless colonies on MacConkey agar, which have a smooth and slimy appearance and a tomb-shaped structure. This observation indicates that the bacterium does not possess the ability to metabolize lactose. The Acinetobacter bacteria grow into pink colonies when cultivated on Leeds Acinetobacter Medium supplemented with specific nutrients. Various Acinetobacter species as nosocomial pathogens: features of resistance and epidemiology (9). The pangenome can reveal genetic variations that are hidden, show the true genetic diversity at the species level, and supplement the genetic information that is lacking based on the examination of a single reference genome. Furthermore, a critical aspect of pangenome

research is analyzing the biological roles of recently identified genes. The identification of non-reference sequences, which frequently come from non-core genomes, by Pangenome has the potential to significantly affect an organism's fitness (10).

1.2   Problem Statement

Acinetobacter species are of significant clinical because they can lead to a variety of infections, they are a matter for concern. and their increasing antibiotic resistance. Understanding the genomic diversity within the Acinetobacter genus is crucial for unraveling the genetic basis of its pathogenicity, virulence, and antibiotic resistance mechanisms. While individual genome sequencing efforts have provided valuable insights, a comprehensive analysis of genomic diversity across various Acinetobacter strains is necessary.

1.3   Aims and Objectives

The purposed of study divided into the following three objectives

1. Isolation, characterization and Antibiotics susceptibility testing of local *Acinetobacter baumannii* isolates.

2. Analysis of Whole genome sequences of isolates with sequences assembly and annotation.

3. Evaluation of genetic diversity and pangenome analysis of *A. baumannii* genome.

Chapter 2

Literature Review

When *Acinetobacter spp.* was identified as Micrococcus calcoaceticus in 1911 after being isolated from a soil sample, research on the species started. On the basis of similar biochemical characteristics, taxonomists did not formally designate the genus Acinetobacter until 1971 (11). Even though this genus has twitching motility and a coccobacillary appearance, its name is derived from the Greek word a-kinetos-bacter, which implies non-motile rod. Thus far, molecular techniques have made it possible to identify more than 65 officially recognized species in the Acinetobacter genus. These bacteria are either non-pigmented or have a pale yellow to gray pigmentation. They are also strictly aerobic, non-fermentative, oxidase-negative, and catalase-positive. The Acinetobacter calcoaceticus–*Acinetobacter baumannii* complex (ACB complex) is made up of closely related species that share similar phenotypic and biochemical characteristics. These species include Acinetobacter calcoaceticus, *Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter seifertii*, and *Acinetobacter dijkshoorniae*, all of which require molecular methods of identification (12). Other than A. calcoaceticus, five other species are linked to human diseases, with *A. baumannii* being the most prevalent clinical species globally. In fact, this opportunistic organism causes bloodstream, urinary tract, skin, and soft tissue infections, as well as nosocomial infections in the community and among critically sick patients in intensive care units (ICUs). It also causes pneumonia linked with ventilators. Unfortunately, there has been a notable rise in the frequency of isolates of *A. baumannii* that are

multidrug resistant (MDR) (13). *A. baumannii*'s genomic plasticity allows it to acquire or upregulate resistance genes in addition to its intrinsic resistance to a number of antibiotics, which limits effective treatment options and raises mortality rates. Apart from possessing genes resistant to antibiotics, *A. baumannii* may possess several other virulence characteristics that enable it to survive in the environment, attach itself to biotic surfaces, infiltrate host cells, and evade the host's immune system (14). Fewer research has concentrated on host–*A. baumannii* interactions, despite the fact that numerous reviews have detailed the mechanisms of antibiotic resistance and the capacity to form biofilms on abiotic surfaces. As a result, the information about the genetic characteristics of *A. baumannii* and the important bacterial elements that both increase its pathogenicity and facilitate its interaction with host cells is summed up in this review. We will also discuss recent studies that have looked into how these virulence factors aid *A. baumannii* in evading the immune system of the human host (15).

2.1   Pathogenic Factor and Virulence Factor

There are comparatively few virulence factors found in *A. baumannii* compared to other Gram-negative pathogens, despite current genomic and phenotypic research uncovering numerous virulence factors responsible for its pathogenicity [16].
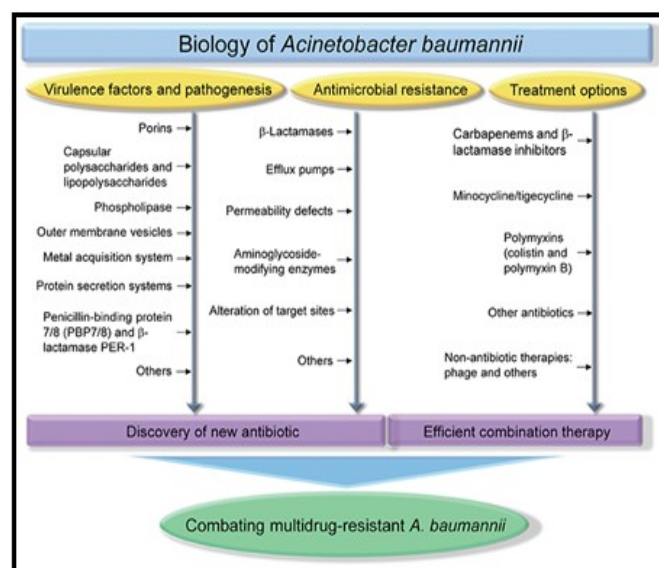
FIGURE 2.1: *A. baumannii* virulence factor and pathogenesis (16)

2.1.1 Porins

Proteins found on the outer membrane called porins are linked to regulating cellular permeability. Porins is the outer membrane is known as OmpA. In vitro model systems have shown links between OmpA, a highly defined virulence component in *A. baumannii*, and several interesting biological characteristics. Human epithelial cells are not susceptible to apoptosis induction by the *A. baumannii* ompA mutant, according to a random mutagenesis screen. Purified OmpA binds itself to the epithelial cells of the host, goes after apoptosis-inducing factor and cytochrome c, two proapoptotic substances, are released by mitochondria and cause apoptosis. By moving through a unique monopartite nuclear location signal to reach the nucleus, OmpA triggers death of cells, according to a different study. By binding to human serum factor H and interacting with fibronectin, Additionally, OmpA is important for epithelial cell adhesion and invasion. This interaction may help *A. baumannii* evade complement-mediated death. Without the ompA gene, *A. baumannii* cannot survive in the mouse lung. (17). Moreover, OmpA contributes to *A. baumannii*'s resistance to antibiotics. OmpA, the main *A. baumannii* porin, has 70 times less pore-forming activity than OmpF. A number of antibiotics, including aztreonam, nalidixic acid, and chloramphenicol, have significant decreases in their minimum inhibitory concentrations (MICs) as a result of the disruption of the ompA gene. These results imply that OmpA couples with inner membrane efflux systems and is involved in the extrusion of antibiotics from the periplasmic space through the outer membrane. OmpA facilitates surface motility and biofilm formation, which enhances *A. baumannii*'s capacity for survival and persistence. Additionally, OmpA controls the synthesis of outer membrane vesicles.These findings imply that the OmpA protein is a desirable target for the creation of cutting-edge antibiotics and prophylactic measures. OmpA may be a helpful vaccination for *A. baumannii*, according to two recent investigations using immuno-proteomics and reverse vaccinology (18). It is accurate to say that patients with invasive *A. baumannii* infections as well as healthy persons respond immunologically to the OmpA protein. When *A. baumannii* was injected into a mouse model, OmpA-injected mice fared far better than control mice in terms of survival (19). Omp33–36, an outer membrane porin

that serves as a water passage channel and is 33–36 kDa, has also been connected to the cytotoxicity of *A. baumannii*. The deletion strain omp33-36 dramatically lowers the cytotoxicity and adhesion of human lung epithelial cells. In a mouse sepsis model, the omp33-36 gene is deleted, which attenuates mortality and lowers bacterial concentrations in the lungs and spleen. According to one study, pure Omp33-36 activates caspases and modifies autophagy to cause apoptosis in many cell types, including immunological and connective tissue cells. In addition, A factor in antibiotic resistance is omp33-36. The antibiotics imipenem and meropenem carbapenem-resistant *A. baumannii* isolate JC10/01 loses Omp33-36. This strain's episomal expression of Omp33-36 evidently lowers the imipenem and meropenem minimum inhibitory concentrations (MICs) (20). While its role in *A. baumannii* pathogenicity is unknown, Additionally, it has been discovered that Omp22 is a unique, conserved, and safe antigen for the creation of vaccinations that successfully impede the growth of *A. baumannii* infections. Omp22 vaccinations, both active and passive, boost mouse survival rates, reduce inflammatory cytokine and chemokine serum levels, and regulate bacterial burdens in peripheral blood and organs. There are additional porins linked to diminished virulence, CarO, or carbapenem-associated outer membrane protein, and OprD-like are two examples.

2.1.2 Polysaccharides Capsular and Lipopolysaccharides

A. baumannii envelope is linked to other pathogenicity-promoting variables in addition to OmpA. LPS and capsular exopolysaccharides are two of these that are pathogenicity factors for *A. baumannii* (21). Interestingly, *A. baumannii* infections have resulted in many isolates from patients carrying the conserved K locus gene cluster. This locus may control the formation of capsular polysaccharides. These isolates also express surface capsular polysaccharides (22). The ptk and epsA genes were found by an arbitrary transposon screening in an exudative proinflammatory fluid to identify genes required for development. It is anticipated that these genes are crucial for the assembly and polymerization of capsules. The growth deficit in human serum of the ptk and epsA mutants, as well as impairments in capsule formation, result in a considerable reduction in

survival at soft tissue infection sites. Mutations in the pglC or pglL gene, which synthesizes the O-pentasaccharide present on glycoproteins and capsular polysaccharides, have been shown to produce septicemia in mice in addition to aberrant biofilm growth and decreased lethality. Consequently, it has been suggested that protective antibody-based therapies should target capsular polysaccharides (23). According to one study, capsular polysaccharides contribute to *A. baumannii*'s resistance to antibiotics. Capsular polysaccharide-deficient mutants exhibit reduced inherent resistance to peptide antibiotics. Furthermore, the overproduction of capsular polysaccharides is brought on by the presence of antibiotics. In a mouse model of systemic infection, antibiotic-induced capsular polysaccharide synthesis improves virulence and resistance to host complement-mediated death. That study also showed that transcriptional increases in K locus gene expression are necessary for enhanced capsule manufacture following antibiotic exposure, and that the two-component regulatory system of the bfmRS regulates K locus gene expression. In a mouse pneumonia model, Inhuman ascites, an ex vivo media that duplicates the infected environment, requires the bfmR gene for lung development and survival. Additionally, adhesion to eukaryotic cells, the production of biofilms, and resistance to human serum are all dependent on BfmS, a virulence factor. The results demonstrated complement-mediated bactericidal activity and BfmR-mediated resistance to both therapeutically significant antimicrobials. That study did, however, imply that BfmR actions are not dependent on capsular polysaccharide synthesis. Consequently, the connection between capsular polysaccharides and BfmRS (23). LPS comprises the majority of the outer layer of membrane of Gram-negative bacteria. This immunoreactive compound functions in a manner that is dependent on toll-like receptor 4 (TLR4), causes macrophages to produce interleukin 8 and tumor necrosis factor. An oligosaccharide core, an endogenous lipid Lipoprotein LPS is composed of a repetitive O-antigen and a moiety. For *A. baumannii* to remain viable and virulent, LPS is necessary. In a model of soft tissue infection in rats, mutant cells lacking LpsB glycotransferase exhibit decreased survival rates and a severely reduced form of LPS glycoform, consisting of only two carbohydrate residues connected to lipid A. These cells also exhibit reduced resistance to human serum. Growth inhibitors do not impede the activity of LpxC, an enzyme that is necessary for the synthesis of lipid A. Inhibition of LpxC completely

protects animals against fatal infection and successfully eradicates *A. baumannii* in a mouse model via boosting the death of opsonophagocytes, decreasing LPS levels in the blood and decreasing inflammation. These findings suggest that preventing the synthesis of LPS is an effective tactic for finding new antibiotics. LPS modification leads to an increase in antimicrobial resistance. Numerous investigations have demonstrated that changes to LPS reduce *A. baumannii*'s resistance to a wide range of clinically significant antibiotics, including colistin (24).LPS comprises the majority of the outer layer of membrane of Gram-negative bacteria. This immunoreactive compound functions in a manner that is dependent on toll-like receptor 4 (TLR4), causes macrophages to produce interleukin 8 and tumor necrosis factor.

2.1.3   Phospholipase

Phospholipase is an essential lipolytic enzyme for the metabolism of phospholipids and an important factor in the pathogenicity of many bacteria, such as P. aeruginosa, Legionella monocytogenes, and Clostridium perfringens (25). Three types of phospholipases have been identified based on the cleavage site: PLA, PLC and PLD. While PLA breaks down the fatty acids from the backbone of glycerol, PLC separates the phospholipid's phosphorylated head group from it. All that PLD, a transphosphatidylase, splits apart in the heads collecting. The host cell membrane's stability is impacted by phospholipid degradation, and disruptions in cellular signaling can be caused by the cleaved head group, which can alter the host immunological response. In *A. baumannii*, It has been discovered that PLD and PLC are pathogenicity factors (26). A1S-0043 and A1S-2055 are the two PLCs of *Acinetobacter baumannii* ATCC17978. *A. baumannii* has a little less cytotoxic effect on epithelial cells than the parental strain when the A1S-0043 gene is inactivated. In a mouse model of pneumonia, one (A1S-2989) of the two PLD genes found in *A. baumannii* strain 98-37-09 is damaged, leading to lower virulence and resistance to human serum, and capacity to infiltrate epithelial cells. A different study found that *A. baumannii* ATCC 196006 carries three PLD genes, all of which work together to contribute significantly to virulence and host cell

invasion. These findings imply that phospholipase enzymes play a crucial role in the pathogenicity of *A. baumannii*. (26)

2.1.4 Outer Membrane Vehicles

OMVs are 20–200 nm diameter, spherical vesicles released by different Gram-negative pathogenic bacteria's outer membranes. They are known to serve as well as delivery systems LPS serves as a vehicle for bacterial effectors entering host cells, phospholipids, periplasmic and outer membrane proteins, and DNA or RNA. Through the simultaneous delivery of several virulence factors to the inside of host cells, OMVs enable pathogen-host interaction without requiring direct bacterial-to-host cell contact (27). Numerous strains of *A. baumannii* secrete OMVs that contain phospholipases, proteases, and OmpA, among other virulence factors. OMVs produced by When interacting with host cells, *A. baumannii* uses lipid rafts to transfer bacterial effectors to the cells, which causes cytotoxicity. Induction of dose-dependent expression of pro-inflammatory cytokine genes in epithelial cells by purified OMVs of *A. baumannii* ATCC 19606. Interestingly, OMVs that have been proteinase-treated don't exhibit an important rise inside the phrase of genes linked to inflammatory-promoting cytokines, stating that the robust innate immune response that OMV membrane proteins stimulate is their own source. According to one study, OMVs have a part in the pathophysiology of *A. baumannii*. When compared to a strain that produces less OMVs, an increased innate immune response and increased cytotoxicity are caused by an *A. baumannii* strain that generates more virulence factors and multiple OMVs (28). Because of the pathogenicity of *A. baumannii* OMVs, multiple trials have demonstrated that *A. baumannii* OMVs could be employed as an acellular vaccine to increase protective immunity. Vaccination with *A. baumannii* ATCC 19606 strain OMVs protects mice against challenge with homologous bacteria and other clinical isolates in a mouse model of disseminated sepsis. Comparable outcomes were seen in a model using pneumonia-stricken rats. Both active and passive OMV immunization dramatically reduced the bacterial burden, inflammatory cell infiltration, and inflammatory cytokine buildup in the pneumonia model.. Based on these findings, it appears that acellular vaccines against *A. baumannii* infections may be

developed using *A. baumannii* OMVs. It's interesting to note that *A. baumannii* OMVs transmit the OXA-24 carbapenemase gene horizontally and are linked to the development of antibiotic resistance (29).

2.1.5 Metal Acquitsion System

Although ferric iron is one of the most abundant elements in biological and environmental systems, its low solubility limit of 1017 M in both aerobic and neutral pH conditions, as well as chelation by low-molecular-weight compounds like heme or high-affinity iron-binding compounds like lactoferrin and transferrin, make ferric iron less readily available to bacteria in its preferred state. Most aerobic bacteria create siderophores, which are high-affinity iron chelators, in order to get around this iron constraint (30). Siderophores are molecules with a modest molecular weight (400–1,000 kDa) that have a strong affinity for iron. Fe3+-siderophore association constants range from 1012 to 1052. Siderophores are classified into three groups: hydroxymates, catecholates as , well as a combination, depending on which moiety provides the oxygen ligands needed to coordinate Fe3+.The capacity of *A. baumannii* ATCC 19606 cells to survive within human alveolar epithelial cells and to infect and kill Galleria mellonella larvae is also significantly reduced by a mutation in the entA gene, which is necessary for the production of the acinetobactin precursor 2,3-dihydroxybenzoic. According to one investigation, MDR *A. baumannii* isolates produce acinetobactin much more frequently than virulent strains (31). The synthesis of Fe-S clusters and the way cells react to oxidative damage and iron chelation are two further aspects of the NfuA Fe-S scaffold protein that have been connected to virulence in *A. baumannii*. Reactive oxygen species (ROS) like hydrogen peroxide and cumene hydroperoxide can damage human epithelial cells more readily and cause the nfuA mutant to proliferate much less in these cells. Furthermore, a model of G. mellonella infection shown that, whilst fewer than 30% of larvae die when infected with the nfuA mutant, over 50% of injected larvae die 6 days after infection with the normal strain. According to one study, iron deficiency boosts PLC synthesis, which raises *A. baumannii*'s hemolytic activity. According to these reports, iron acquisition functions are essential for the pathogenicity of *A. baumannii* (32). Metals like zinc (Zn2+ and

Zn) and manganese (Mn2+ and Mn) are chelated by the innate immune protein calprotectin, stops bacteria from growing. In vivo, *A. baumannii* can induce illness when this nutritional immune protein is present. *A. baumannii* utilizes a zinc acquisition system (ZnuABC) that is up-regulated in environments to fight zinc restriction where zinc is scarce, and the znuB mutant strain starves of zinc during concentrations higher than those of the normal form. ZnuB has a role in the lung infection pathogenesis caused by *A. baumannii*. Notably, in multidrug-resistant *A. baumannii*, a zinc deficiency lowers the imipenem minimum inhibitory concentration (MIC) in *A. baumannii* to below the imipenem resistance clinical breakpoint. This may be because a large number of carbapenemases are metalloenzymes that need zinc (Zn) in order to hydrolyze. *A. baumannii* has been shown to harbor the unique Zn metallochaperone ZigA in addition to the ZnuABC system. ZigA has a close relationship with zinc, which is necessary for both bacterial growth in the presence of zinc deprivation and mouse disseminated infection (33). The method that *A. baumannii* uses to get around a Mn restriction has been found. When *A. baumannii* is exposed to calprotectin-induced Mn deprivation, it enhances the transcription of a Mn transporter belonging to the urea carboxylase and the NRAMP (Natural Resistance-Associated Macrophage Proteins) family to negate the antibacterial properties of calprotectin. Two proteins appear to be virulence factors: In a murine pneumonia model, the fitness of *A. baumannii* is increased by an NRAMP family transporter (MumT), and When calprotectin is present, MumC is the urea carboxylase enzyme essential for the growth of *A. baumannii*. MumC depends on *Acinetobacter baumannii*'s ability to use urea as its only supply of nitrogen. The authors hypothesize a link based on MumC's contribution to the calprotectin-resistant *A. baumannii*, between metabolic stressors such nitrogen stress and metal starvation famine (27).

2.1.6 Protein Secretion System

In *A. baumannii*, many protein secretion mechanisms have been found. Type II secretion system (T2SS) the most recent characterized System of secretion for *A. baumannii*. T2SS is a complex of several proteins that has several structural characteristics with type IV pili systems, a typical appendage of Gram-negative

bacteria. A large variety of proteins are translocated by T2SS from the cell's surface or the periplasmic region into the extracellular milieu. The T2SS is composed of four sub-assemblies: the outer-membrane complex with dodecamers, the pseudopilus, the assembly of the inner membrane platform, and the ATPase for cytoplasmic secretion, which is made up of 12–15 proteins (14). T2SS secretion involves two steps procedure. First, target proteins are moved to the periplasm by the twin arginine transport (Tat) or general secretory (Sec) systems, from which point the T2SS secretes the target proteins out of the cell. LipA secretion is eliminated when the *A. baumannii* genes encoding gspD or gspE, the T2SS components, are deleted, showing that LipA serves as a substrate for T2SS. In a neutropenic mouse model of bacteremia, lipA, gspD, and gspE mutant bacteria grow poorly in vivo because lipase breaks down long-chain fatty acids. Long-chain fatty acids cannot support their growth as their exclusive carbon supply.*A. baumannii*'s complete virulence possesses demonstrated in lung infection models in mice and G. mellonella, which highlight the importance of a functional T2SS. Metalloprotease CpaA and the lipases LipA, LipH, and LipAN are examples of T2SS substrates. Interestingly, of these secreted proteins, two (LipA and CpaA) require certain chaperones in order to secrete. When these chaperones are inactivated, LipA and CpaA secretion is stopped. They are encoded next to their corresponding effector (6). *Acinetobacter baumannii* also has a type VI secretion system (T6SS). First detected in P. aeruginosa and Vibrio cholera, the T6SS was found. In order to gain an edge in colonization during the infection of eukaryotic hosts or to eliminate rival bacteriaa large number of bacteria inject effector proteins using the T6SS (34). In V. cholera, the T6SS causes DNA release and horizontal gene transfer, which could contribute to the development of antibiotic resistance. The T6SS possesses a contractile bacteriophage sheath-like structure that forms a structural needle or spike that is employed to enter the targeted cell. Type II secretion System of secretion for *A. baumannii*.It is made up of numerous conserved structural proteins and auxiliary components. VgrGs attaches effector domains to the spike, the structural protein Hcp produces a polymerized tubular structure that is released from the cellular, and the distinctive needle's sharp tip -like shape is formed by the PAAR repeat protein: proline-alanine-alanine-arginine (20).

2.1.7 PBP7/8 (Penicillin-Binding Protein) and PER-718 (β-Lactamase)

Even though PBPs are frequently associated with B-lactam antibiotic resistance, the pathogenicity of *A. baumannii* is enhanced by PBP7/8, which is produced through the pbpG gene. The pbpG mutant strain develops similarly to the the normal form strain in Luria-Bertani medium; however, it grows less in human serum and exhibits much worse survival in models of pneumonia and soft-tissue infection in rats. A study utilizing electron microscopy to examine the morphology of bacteria revealed that the absence of PBP7/8 could have impacted the structure of peptidoglycans, potentially influencing the susceptibility to host defense mechanisms (35). It's interesting to note that B-lactamase PER-1 may contribute to *A. baumannii* pathogenicity. PER-1 is associated with cell adhesion despite being an extended-spectrum B-lactamase (ESBL). Although all strains that are PER-1-deficient exhibit adverse cell adherence, nine strains that produce PER-1 Links to the Caco2 cell lines. Interestingly, pathogenicity in a wide range of bacteria, including K. pneumoniae, P. aeruginosa, and E. coli, has been associated with several B-lactamases. On the other hand, no universal processes have been suggested (34).

2.1.8 Others

Acinetobacter Serum resistance is mediated by a new plasminogen binding and complement inhibitory protein called CipA. *A. baumannii*'s serum resistance is partly attributed to the conversion of plasminogen that binds to CipA to active plasmin, which complements C3b and breaks down fibrinogen. Consequently, the cipA mutant strain exhibits a deficiency in endothelium monolayer penetration and is effectively destroyed by human serum. The prolonged factor for translation Tuf, like CipA, belongs to the plasminogen-binding protein from *A. baumannii*. By means of proteolysis, Plasminogen that binds to tuf can be transformed into active plasmid, that breaks down fibrinogen and component C3b. RecA is a virulence factor for *A. baumannii* that is involved in the SOS response and homologous recombination. Within a mouse model of systemic infection, the recA mutant

exhibits markedly decreased mortality and considerably decreased survival inside macrophages. Surface antigen protein 1 (SurA1) has a tremendous impact on the pathogenicity and fitness of *A. baumannii*. There is a significant decrease in serum resistance between the surA1 mutant and the CCGGD201101 wild-type strain. The insect model of G. mellonella exhibits decreased spread and a worse survival rate for a surA1 mutant strain (36). A study on the growth of *A. baumannii* transposon mutants: 250 000 revealed the requirement of G. mellonella larvae possess 300 genes necessary for *A. baumannii* survival or development. The 300 genes were divided into six groups: transcriptional regulation, stress response genes, antibiotic resistance, cell envelope/membrane/wall, aromatic hydrocarbon metabolism, cysteine metabolism/sulfur assimilation, and micronutrient acquisition. Gig (growth in Galleria) genes are four transcriptional regulators that are necessary for the growth of G. mellonella larvae. These genes (gigA-D) were lost, and the result was a severe deficiency in G. mellonella larvae growth and mortality. Stress proteins like UspA were found to be necessary for G. mellonella proliferation in this study. According to a different study, UspA is crucial to the pathophysiology of *A. baumannii* sepsis and pneumonia. Aromatic hydrocarbon metabolism involves some of the 300 genes. According to a different study, GacS, a transcription-related protein that controls the gene expression similar to paaE and controls the phenylacetic acid catabolism pathway, has an impact on the virulence of *A. baumannii*. The importance of aromatic hydrocarbon metabolism in *A. baumannii* pathogenicity was verified by experiments with a paaE deletion mutant, while the molecular mechanism is yet unknown. Surprisingly, a recent study revealed that *A. baumannii* phenylacetate accumulation stimulates bacterial clearance by inducing a rapid neutrophil inflow to a particular infection site. They postulated that phenylacetate functions as a neutrophil chemoattractant, initiating bacterially-directed neutrophil chemotaxis. This work implies that the phenylacetic acid catabolic pathway may have a unique molecular function in the pathogenicity of *A. baumannii* (37). *A. baumannii* uses biofilm formation to evade the immune system, and pili are necessary for the bacterium's pathogenicity and adhesion to abiotic surfaces where biofilm formation occurs. Significant genes required for type IV pili synthesis are expressed when the imipenem-resistant

A. baumannii isolate is treated with imipenem; this suggests that *A. baumannii* has a biological advantage because of its ability to overproduce pili. Among the additional virulence-related proteins that have been found are the type of membrane transporter AbeD is resistance-nodulation-division, OmpR/EnvZ, and FhaBC; however, their molecular mechanisms remain unclear (4).

2.2 Genome Plasticity, Genomics, and Pan-Genomics

As *A. baumannii* infections multiplied, it became evident right away that isolates had remarkably different antibiotic profiles. Several research have concentrated on *A. baumannii* isolates' genotypic and phenotypic characteristics because of the elevated prevalence of diseases worldwide and the dearth of medications for types of bacteria resistant to drugs. To look into the connections between *A. baumannii* isolates, two multilocus sequence typing (MLST) strategies were used. The expansion and dispersion of many bacterial lineages were emphasized by these investigations. Owing to local expansions of *A. baumannii* isolates, MLST demonstrated a unique geographic distribution for each lineage. Out of nine international clones (ICs) that have been discovered thus far, IC-I and -II currently lead the way in terms of pandemics across continents (19). According to a different study, UspA is crucial to the pathophysiology of *A. baumannii* sepsis and pneumonia. Aromatic hydrocarbon metabolism involves some of the 300 genes. According to a different study, GacS, a transcription-related protein that controls the gene expression similar to paaE and controls the phenylacetic acid catabolism pathway, has an impact on the virulence of *A. baumannii*. The importance of aromatic hydrocarbon metabolism in *A. baumannii* pathogenicity was verified by experiments with a paaE deletion mutant,Although MLST has benefits, not enough information has been gathered to ascertain the genetic connections amongst the rise in isolated cases of *A. baumannii*. Novel technology for sequencing have led to significant advancements in our understanding of whole genomes. This approach allowed for the comprehension of the level of genetic variation among *A. baumannii* isolates with regard to insertions, deletions, inversions, and single nucleotide

polymorphisms (38). The significant genetic variations are a result of *A. bauman-nii*'s highly plastic genome, which permits the removal of unwanted genes and the acquisition of external genetic material mostly through allelic recombination and horizontal gene transfer in particular hotspots (13). Taking into account environmental strains that are primarily isolated from hospital inert surfaces and pathogenic strains that are linked to human hosts, these results support the theory because of their high level of gene variety, *A. baumannii* isolates are better able to adapt to novel habitats; genetic variations are driven by the bacterial environment or pathogenic lifestyle. In an attempt to understand more about how *A. baumannii* has adapted to specific settings, the majority of study has focused on the pangenome of the organism. The core and auxiliary genomes are collectively referred to as the "pangenome" The accessory genome comprises genes that were detected in only one or a small number of strains (unique gene families), whereas the core genome describes the genes that are shared by all strains in the investigation. According to a recent study, the pangenome of *A. baumannii* harbors about 19,000 distinct genes, although the core genome of the species is predicted to include about 2200 genes [45]. *A. baumannii* groupings according to a study of the genes that 2467 genomes have in common. The first group, which makes up 34% of the strains studied, is characterized by prokaryotic toxin-antitoxin systems (TASs), rare plasmids, Genes related to the restriction-modification system (rms) or CRISPR-associated (cas) (39). Given that TASs have an impact on apoptosis, cell cycle processes, and metabolic processes within cells, they are significant virulence factors in adverse environmental settings (26). In contrast, a greater count of genes designated as genes with exonucleases in plasmids were shared by the second group, which comprised 66% of the examined *A. baumannii* strains, whereas a smaller count of genes involved in biofilm formation were discovered. CRISPR/Cas systems have been shown to negatively correlate with isolates of *A. baumannii* that are multidrug resistant and to slow the rate at which antibiotic resistance genes are acquired, according to recent studies (31). Thus, it is possible to postulate that CRISPR/Cas systems, which favorably impact biofilm formation to increase perseverance as well as prevent the absorption of foreign DNA, such as genes linked to antibiotic resistance, are what distinguish environmental strains. On the other hand, strains connected to humans lose their CRISPR/Cas systems,

which facilitates their evolution into MDR strains and allows them to survive in environments rich in antibiotics. Moreover, isolates from contaminated samples demonstrate a collection of genes necessary for the production of acinetobactin when iron acquisition systems are taken into account, while environmental samples lack the same set of genes, which have genes involved in xeno-siderophores' ability to absorb iron (40). Due to the significant genomic plasticity of *A. baumannii* strains, there is generally more variability among them the more data obtained from their genome sequencing. This approach revealed the genomic divergence of strains related with the environment and humans, even if the increased number of healthcare isolates threw bias into the genome sequencing data. It appears that the distinct gene pool found in each strain is the result of genetic alterations brought about by niche variations. Additional genome comparison studies comparing isolates, both clinical and nonclinical will contribute to our knowledge of the evolution of *A. baumannii*, the dissemination of antibiotic resistance, and the persistence of nosocomial infections (12).
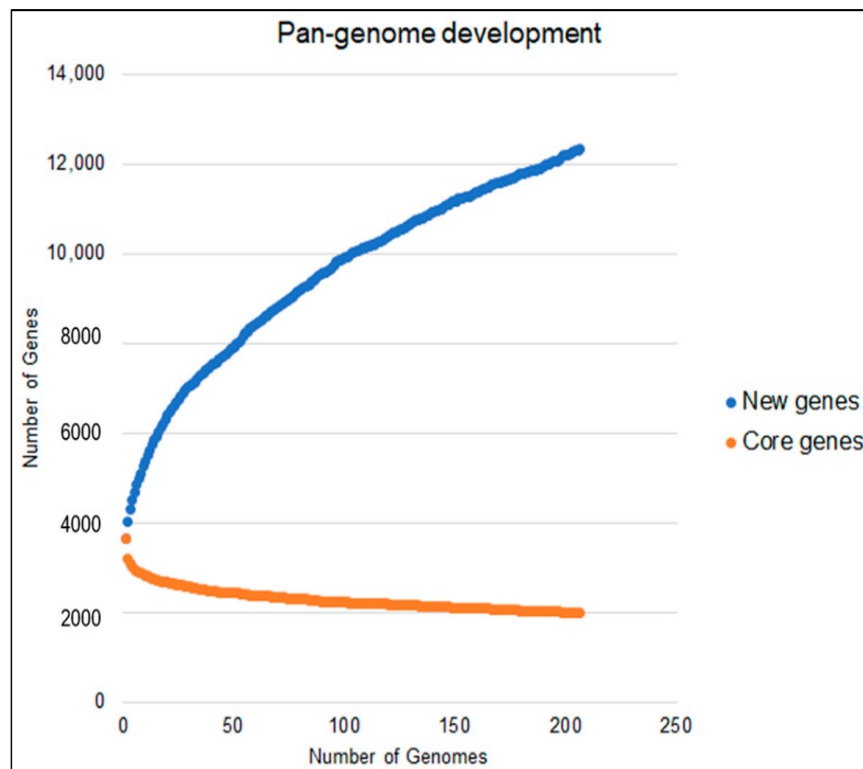
FIGURE 2.2: *Acinetobacter baumannii*'s pan-genome development curve (31).

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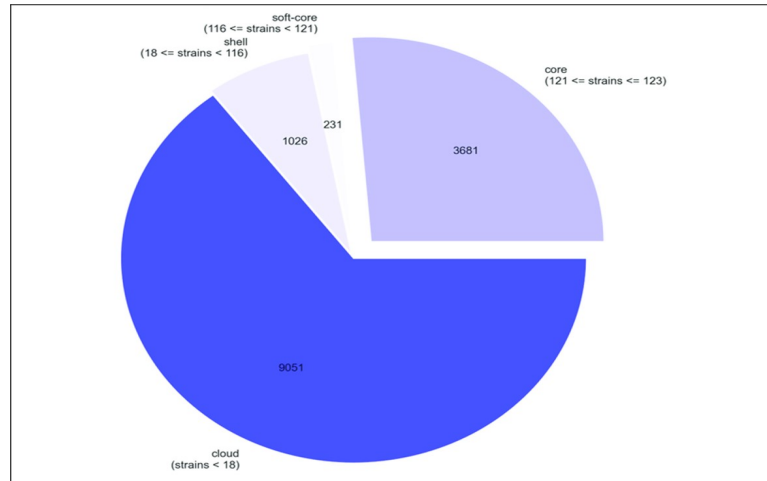
FIGURE 2.3: *Acinetobacter baumannii*'s pangenome represented as a pie chart. The percentage of core and accessory genes every is displayed in the chart (40).

2.3 Clinical Importance

About 2 % of nosocomial infections in the US and Europe are caused by *A. baumannii*, which is twice as common in Asia and the Middle East. Overuse of antibiotics without moderation has been associated with drug resistance to all currently prescribed antibiotics. If innovation in treatment approaches is not pursued, the pre-antibiotic era will soon recur. B -lactamase synthesis, target site modification, permeability deficits, enzymes that modify aminoglycosides, and efflux pumps are just a few of the tactics *A. baumannii* uses to evade the effects of antibiotics. Gram-negative bacteria such as Pseudomonas aeruginosa and Klebsiella pneumoniae are much more common than *A. baumannii* infections, which are comparatively rare. However, multidrug resistant phenotypes are alarmingly four times more common than *A. baumannii* infections. At first, isolates of *A. baumannii* were susceptible to carbapenems. However, up to 90% of *A. baumannii* infections are resistant to carbapenems, according to the World Health Organization's 2016 Surveillance of Antimicrobial Resistance: Annual Report for Central Asia and Eastern Europe. Studies conducted in Europe, the Eastern Mediterranean, and Africa revealed that *A. baumannii* was resistant to carbapenem and caused 20.9%, 13.6%, and 13.6 % of nosocomial infections, respectively. Because

of its mutability, capacity for horizontal gene transfer, and outer membrane vesicles, *A. baumannii* has developed into strains of the fungus that are extensively drug-resistant (XDR), pan-drug-resistant (PDR), and multidrug-resistant (MDR).

2.4 Interactions between the Respiratory Epithelium and Host-*A. baumannii*

The concerning increase in antibiotic-resistant isolates has led to an increase in research on *A. baumannii*'s pathogenicity. Since ventilator-associated pneumonia is so common, in vitro and in vivo pulmonary models have been the focus of most studies. The respiratory mucosa is made up of the lamina propria, basal lamina, and epithelium. The final two offer structural and metabolic support to nearby cells and make up the extracellular matrix (ECM). The extracellular matrix (ECM) is composed of many various proteins, such as vitronectin, fibronectin, laminin, elastin, and collagen (41). Rather, type I and type II alveolar cells shape the alveolar epithelium; cells of type I, together with the underlying endothelium, are in charge of gas exchange, while type II cells produce proteins known as surfactants and guard opposing airborne infections (42). It is made easier for pathogenic bacteria like *A. baumannii* to make physical contact with host cells by the expression of receptors by alveolar cells and extracellular matrix (ECM) proteins. The first proof that *A. baumannii* may attach itself to host epithelial cells and infiltrate them [52]. It was shown by in vitro studies that a cellular receptor-dependent zipper-like mechanism internalizes *A. baumannii*.; after internalization, the bacterial cells are housed in membrane-bound vacuoles. The main role of the major porin OmpA was emphasized by Choi et al. and was the subject of multiple subsequent investigations. In fact, it has been demonstrated that OmpA obstructs cell autophagy (43). Through the process of autophagy, damaged cytoplasmic proteins, organelles, and certain phagocyted bacteria are eliminated by being transported to lysosomes. This complex process requires several proteins, such as beclin 1, microtubule-associated protein light chain 3 (LC3), p62, Rab-7, Rab-5, and autophagy-related proteins (ATGs). An *A. baumannii* OmpA stimulates the c-Jun N-terminal kinase (JNK) signaling pathway, which causes the

macrophage and epithelial cell lines (Hela and RAW264.7, respectively) to undergo autophagy. Furthermore, they have observed an inhibition in the breakdown of p62 and an increase of the lipidated form of LC3 (LC3BII) (38). Furthermore, OmpA inhibits the fusion of autophagosomes with lysosomes, resulting in an incomplete autophagy that permits *A. baumannii* bacterial cells to survive and remain inside the autophagosomes. Due to insufficient autophagy, cells increase the production of IL-1B, an inflammatory cytokine, which triggers an inflammatory reaction throughout the body and may facilitate the spread of bacteria to nearby tissues. These findings were confirmed in further detail and expanded to include porin Omp33 [54]. The release of OMVs, which are made up of OmpA and Omp33, is a crucial aspect of *A. baumannii*'s infectious process. In both in vitro and in vivo settings, imported OmpA has been shown to concentrate through the activation of host mitochondrial GTPase Dynamin-Related Protein 1, ultimately leading to cell death by mitochondrial fragmentation and an increase in ROS generation (25). Additionally, the T5cSS Ata protein showed adherence to host cells. It attaches itself to laminin molecules and collagen I, III, IV, and V by the use of four SVAIG and an RGD motif. In fact, in vivo models revealed a diminished virulent behavior in ata mutants. ECM serves as a host structure for adhering bacteria as well. Epithelial cells interact with the extracellular matrix (ECM) using integrins, cadherins, selectins, and members of the immunoglobulin cell adhesion molecule superfamily (IgCAMs)(44). This protein, T5bSS FhaB *A. baumannii*, is remarkably capable of binding both host integrins and fibronectins. It also has an RGD motif. The majority of integrins specifically detect and bind to the RGD motif, which is found in numerous Collagen I, fibronectin, and laminin are examples of ECM proteins. This accounts for the domain's presence in *A. baumannii* adhesin. As a result, when integrins attach to specific bacterial adhesins, host cells' internal signaling pathways are triggered. This remodeling of the actin cytoskeleton causes the bacteria to internalize (45).

Numerous virulence characteristics of *A. baumannii* allow the bacteria to attach to and infiltrate host cells.Via a zipper mechanism, *A. baumannii* cells are internalized and live inside membrane-bound vacuoles. When *A. baumannii* infections occur, bronchial epithelial cells react by activating the intracellular oxidative stress
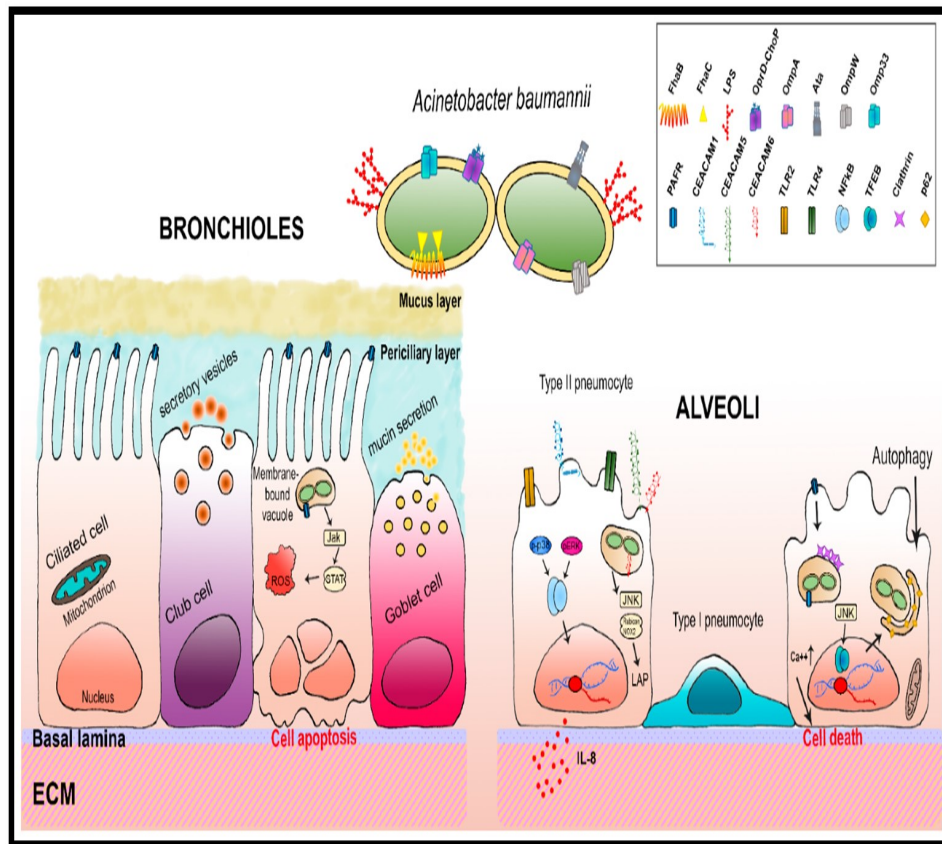
FIGURE 2.4: This shows the Diagrammatic depiction of the interactions between the respiratory epithelium and the bectria *Acinetobacter baumannii*

response and the Jak-STAT pathway, which ultimately results in apoptosis. Type II pneumocytes with TLR2 and TLR4 are able to detect lipoproteins and LOS. They then use an p38-Erk1/2-dependent pathway and NF-kB release cytokines, such as IL-8, which chemoattract neutrophils to the infection site (41). *A. baumannii* can access type II pneumocytes via interacting with CEACAM-1, -5, and -6. Through an NF-kB and Erk1/2-dependent pathway, internalization through CEACAM-1 causes TLR2, TLR4, and IL-8 production. That being said, IL-8 secretion decreases significantly 24 hours after infection, potentially owing to the TLR2 signaling cascade's blockage by CEACAM-1 ITIM by bacteria. On the other hand, when CEACAM-5 and -6 are engaged, the route JNK1/2-Rubicon-NOX2 is inhibited, leading to *A. baumannii* removal and LC3-associated phagocytosis (LAP) [108]. Additionally, through the ChoP-containing OprD, In order to initiate a signaling cascade that includes clathrin, B -arrestins, G protein-coupled PLC, and an increase in intracellular Ca++ that eventually leads to bacterial internalization, *A. baumannii* can interact with platelet-activating factor receptors

(PAFRs) (46). TFEB facilitates *A. baumannii*'s invasion and persistence by inducing the autophagosome-lysosome system, which the bacterium uses to move intracellularly and survive in lung cells—possibly as a result of decreased acidification of lysosomes. There is a theory that *A. baumannii* employs both apoptosis and intracellular retention as means of spreading to deeper tissues and causing invasive illnesses. Parts separately are not scalable (4).

2.4.1 TLRs

Sentinel lymphocytes (TLRs), which are able to identify specific components of bacteria or disease-associated molecular patterns (PAMPs), serve as markers for the diagnosis and treatment of microbial infections.TLRs thereby initiate signaling pathways intended to eliminate infections upon detection of their particular microbial components (47). Of the ten human TLRs that have been discovered so farTLR4 identifies lipopolysaccharide (LPS), TLR9 is activated by unmethylated CpG containing ssDNA, and TLR2 attaches to bacterial lipoproteins [102]. As an experimental paradigm, the A549 cell line is utilized to show that TLR2 and TLR4 are able to identify *A. baumannii*. Via MAPKs p38 and extracellular signal-regulated kinase (ERK), these receptors connect to and activate nuclear factor-kappa B (NF-kB), which in turn causes the release of interleukin-8 (IL-8)50%. Both in vitro and in vivo, IL-8 secretion is essential for lung neutrophil inflow and *A. baumannii* clearance (48). TLR9 is different from other TLRs in that it is found inside of endosomes. Using tlr9 -/- knockout mice, its role in *A. baumannii* infections was discovered; infected animals showed increased extra-pulmonary bacterial spread and more severe lung lesions than wild-type mice.transcription factor EB (TFEB) using the A549 cell line. TFEB causes up to 79 autophagic genes to be upregulated. One of these genes is LC3BII, which together with p62 leads to the intracellular movement of *A. baumannii* via the autophagosome-lysosome system and its persistence in lung cells, Interestingly, similar results were shown in an animal model of aged mice that were TLR9-competent; this led to the conclusion that TLR9 expression is essential for the elderly's immune response against *A. baumannii*-caused lung infections (49).

2.4.2   PAFRs

An additional family of surface-exposed receptors that detects platelet-activating factor (PAF), a powerful phospholipid mediator, physiologically, the G-protein-coupled seven-transmembrane receptor PAFR is a pro-inflammatory mediator, which is found on the respiratory system's epithelial cells, among other tissues. But because PAF and bacteria share phosphorylcholine (ChoP) moieties, PAFR attaches itself to bacterial ChoP, permitting the germs to adhere to and infiltrate the respiratory epithelium. Furthermore, binding to PAFR initiates an endocytic process that involves clathrin, B-arrestins, and G-protein-phospholipase C. By this pathway, both in vivo and in vitro, *A. baumannii* internalizes lung epithelial cells (46). Furthermore, Parra-Millán et al. showed that *A. baumannii* infections activate the transcription factor EB (TFEB) using the A549 cell line. TFEB causes up to 79 autophagic genes to be upregulated. One of these genes is LC3BII, which together with p62 leads to the intracellular movement of *A. baumannii* via the autophagosome-lysosome system and its persistence in lung cells, most likely due to a reduction in lysosome acidification. More recently, it has been demonstrated that the entry of *A. baumannii* ChoP-PAFR into human bronchial epithelial cells can cause intracellular oxidative stress, apoptosis, and the activator of transcription and Janus kinase (Jak)-signal transducer in response to bacterial infections (19).

2.4.3   CEACAMs

CEACAM receptors are a class of glycoproteins connected to immunoglobulin (Ig) that are implicated in several biological processes and to which respiratory infections can bind, including inflammation, cancer progression, intracellular and intercellular signaling, and cell adhesion. When *A. baumannii* engages these receptors, two distinct CEACAM-dependent signaling pathways are triggered. IL-8 is first secreted by the CEACAM-1-dependent route through Erk1/2 and NF-kB signaling; however, after 24 hours of infection, IL-8 levels sharply decrease. On the other hand, LC3 associated phagocytosis (LAP) is triggered by CEACAM-5 and -6. The quick advancement of model organisms, animal models, and in vitro

cell cultures will broaden our understanding of the molecular interactions of *A. baumannii* during lung infections (50).

2.5 Whole-Genome Sequencing (WGS)

The WGS method gathers the nucleotide sequence of an organism's entire genetic makeup. Haemophilus influenzae was the first bacterium to have its whole genome sequenced when it was annotated and its genome sequenced in 1995 (Fleischmann et al. 1995). This massive project took several years as well as a huge budget to complete. In the following two decades, next-generation sequencing (NGS) rapidly improved and became the most acceptable approach for genomic researchers. NGS is a high-throughput technique and capable of producing sequencing reads of millions of DNA strands that may be joined together to produce a complete genome of nucleotide sequence. As a result, the sequencing capacity is greatly increased, and reduced turnaround time and cost of sequencing compared to Sanger sequencing. When *A. baumannii* engages these receptors, two distinct CEACAM-dependent signaling pathways are triggered. IL-8 is first secreted by the CEACAM-1-dependent route through Erk1/2 and NF-kB signaling; however, after 24 hours of infection, IL-8 levels sharply decrease.In the last decade, NGS technology has improved significantly in terms of processing time and cost with sequencing capacity doubling every 8 to 10 months (27). In consequence of these technological advancements, WGS has become a much more accessible technique for researchers to investigate all aspects of microorganisms. WGS has proven to be an extremely specific and discriminatory molecular approach for investigating clinically important pathogens because it offers data at SNP level throughout a complete genome (16). WGS is currently being used to infer the genetic epidemiology of isolates on a global scale, not just in limited hospital settings. Furthermore, WGS data can be used to obtain MLST, spa typing, SCCmec typing, virulence factors and antibiotic resistance data, offering extra depth of detail from a single approach (47)

2.6   Biofilm Development

A biofilm is a complex, three-dimensional arrangement that arises when microbial cells cling to either living or non-living surfaces due to a combination of physiological and environmental factors, some of which remain undiscovered. Moreover, these biological entities undergo continual proliferation and generate extracellular polymeric substances (EPS), resulting in the formation of a matrix that envelops these microorganisms. The process of biofilm generation and development encompasses five primary stages (5). Medical equipment that are contaminated can introduce germs that lead to biofilm-associated disorders. Examples of these devices include intravascular catheters, cardiac devices, prosthetic joints and shunts, and prosthetic vascular grafts. These infections may arise on their own from native valve endocarditis, dental plaques, or open wounds. *A. baumannii* possesses the capacity to generate biofilm on medically significant surfaces, enabling its persistence within the hospital milieu. This microorganism is the principal cause of several infections, including urinary tract infections (UTIs), meningitis, pneumonia, and bacteremia, among other illnesses that are seen in severely ill patients in intensive care units (ICUs) in hospitals. When compared to individual planktonic cells, these infections show a markedly increased resistance to antibiotics and are associated with the production of biofilms. Bacterial cells are protected from the effects of antibiotics and bacteriophages by the biofilm matrix, which also allows the cells to survive in harsh conditions like desiccation. The potential of *A. baumannii* clinical isolates to form biofilms and drug resistance patterns have been studied in a number of investigations. As previously shown, *A. baumannii* isolates with higher resistance levels show a reduced ability to form biofilms, whereas isolates with lower resistance levels typically produce more durable biofilms. Thereafter, research revealed that strains exhibiting resistance possess the capacity to generate biofilms of moderate to high intensity. Conversely, susceptible isolates initially demonstrated the ability to develop robust biofilms during a 24-hour timeframe, but thereafter saw a reduction in their biofilm-forming capability, resulting in the formation of weaker biofilms. Based on information from the Centers for Disease and Prevention and the National Institutes of Health, biofilms are thought to be involved in 65–80% of human bacterial infections (15). These conditions

have also led to serious economic problems because of product adulteration, energy waste, equipment damage, and the spread of infections. Biofilm-associated infections in tissues present diagnostic obstacles as well as therapeutic difficulties since non-invasive imaging approaches cannot identify the causative bacterium. Furthermore, biofilms can promote the affiliation and colonization of surrounding bacteria by exerting control over them, hence inducing polymicrobial activity.All of these components, which makes choosing an effective empirical antibacterial treatment very challenging. Ultimately, the treatment approach calls for surgically removing implants or grafts that have biofilm-associated infections (39).Based on information from the Centers for Disease and Prevention and the National Institutes of Health, biofilms are thought to be involved in 65–80% of human bacterial infections .Infections because waiting too long to receive the right treatment might have a negative impact on a patient's health. Though useful in the short term, this prescribing approach compromises the medications' future potency as potent antibacterial agents.This microorganism is the principal cause of several infections, including urinary tract infections (UTIs), meningitis, pneumonia, and bacteremia, among other illnesses that are seen in severely ill patients in intensive care units (ICUs) in hospitals.
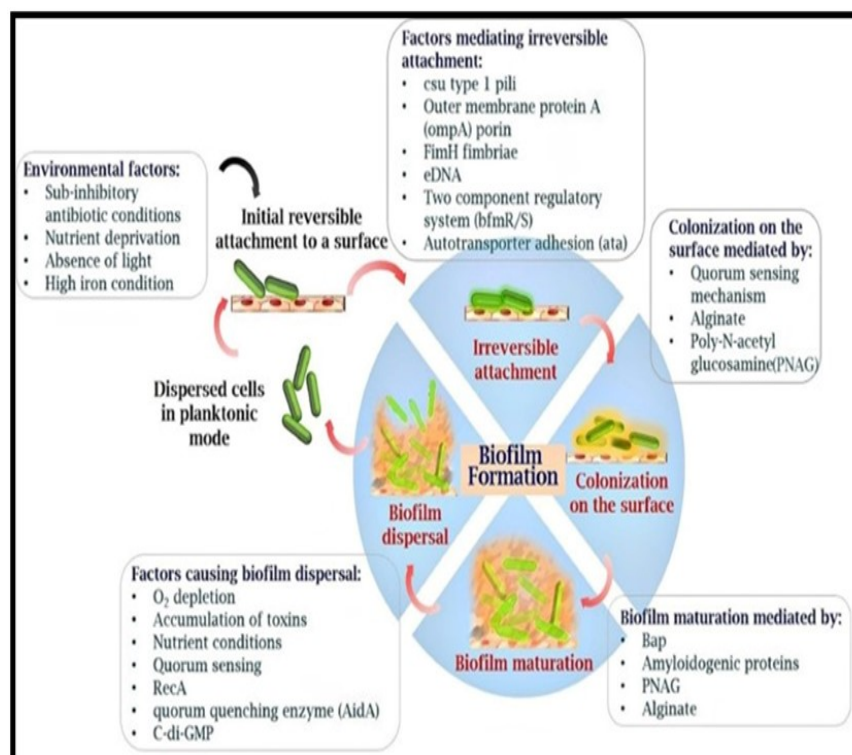

FIGURE 2.5: shows the biofilm formation of *Acinetobacter baumannii* (41).

2.7 Therapeutic Strategies

2.7.1 Existing Antimicrobials

In addition to the several acquired antibiotic resistance pathways that set *A. baumannii* apart, this review does not address them but comprise enzymes that break down B -lactams, alter aminoglycosides, change quinolone binding sites, modify various efflux mechanisms, and alter outer membrane proteins. A highly drug-resistant disease can be created by combining any or all of these components, which makes choosing an effective empirical antibacterial treatment very challenging. In fact, treatment of the illness should be carried out after careful consideration of antimicrobial susceptibility tests, given that one of the primaries first-line antibiotics is probably resistant to *A. baumannii*. Nonetheless, carbapenems like imipenem are frequently prescribed as the medicine of choice for serious and suspected Acinetobacter infections because waiting too long to receive the right treatment might have a negative impact on a patient's health. Though useful in the short term, this prescribing approach compromises the medications' future potency as potent antibacterial agents (31).

2.7.2 Future Therapies.

A. baumannii control tactics have been developed in a number of ways, including but not limited to the following, in response to the threat posed by the rapid and widespread development of antibiotic resistance. Phage-based. Recently, there has been a renewed focus on the treatment of phage infections caused by antibiotics.A highly drug-resistant disease can be created by combining any or all of these components, which makes choosing an effective empirical antibacterial. Because phage has such high specificity and speed, bacteriophage therapy is being reexamined as a viable alternative medicine can aid in addressing the antibiotic resistance issue. The virulent AB1 bacteriophage was isolated and characterized, and it has been shown to be effective against *A. baumannii*, suggesting the possibility of a new treatment (51).

2.7.3 Bactericidal Gene Transfer Therapy

In order to incorporate bactericidal genes into target pathogenic organisms, attenuated donor cells are utilized in the creation and delivery of vectors for bactericidal gene transfer treatment. Positive results have been observed utilizing mouse burn infection models, despite the fact that this approach's therapeutic potential is restricted by the need for donor cells to come into contact with the pathogen in order to initiate vector transfer. By using this method, it was demonstrated that mice that received a single dosage of 1010 CFU of donor cells harboring bactericidal genes had less *A. baumannii* in their burn wounds (30).

2.7.4 Cathelicidins

With an advanced lactation system, animals give birth to immunologically immature, altricial young that stay in the mother pouch for nine to ten months. Cathelicidins destroy both Gram-positive and Gram-negative bacteria, protozoa, and fungus inside the pouch by electrostatically interacting their positively charged peptides with the negatively charged substances present in their targets' cell membranes.Human LL-37 is the most researched cathelicidin since it is the only one with anti-HIV and anti-tumor qualities. Research has demonstrated that the Tammar Wallaby cathelicidin WAM1 is 3-80 times more potent than LL-37 in the treatment of several bacterial infections, such as Acinetobacter. Human red blood cells cannot be hemolyzed by WAM1, so it can be given parenterally to people.Given its ability to withstand salt concentrations comparable to those seen in the human body and its anti-microbial characteristics, WAM1 appears to be a promising option for more in vivo studies (36).

2.7.5 Radioimmunotherapy.

As fast and effective as cancer cells, radioimmunotherapy can target bacteria, despite not being used as a medicinal antibacterial technique within the clinic yet. This method delivers radionuclides that emit deadly levels using the specificity of

antigen-antibody interactions to deliver lethal radiation directly to the target cell. Treatments for bacterial, fungal, and viral infections have been effectively adapted to radioimmunotherapy, which only causes temporary hematological damage in experimental animals. It is certainly possible to employ radioimmunotherapy as a unique therapeutic approach for *A. baumannii*, as prior research has previously documented the production of antibodies against the fungus (11).

2.7.6   Photodynamic Therapy.

involves combining oxygen and visible light with nontoxic photosensitizers (PSs) to create reactive oxygen species, which oxidize biomolecules and kill cells. When treating localized bacterial infections, photodynamic therapy (PDT) usually entails applying a topical PS to the affected area and then illuminating it with red (or near-infrared) light that can penetrate the tissue. Using a mouse burn wound model, this approach has already been shown to be effective against *A. baumannii*, with no discernible impact on wound healing. Chitosan, a polycationic biopolymer, has been shown recently to increase PDT's effectiveness against a variety of infections(19), including *A. baumannii*. The bacteria were totally eliminated after 30 minutes of treatment with chitosan (0.025%) under circumstances where At an initial inoculum of 108 CFU/ml, hematoporphyrin-PDT showed a bacteriocidal effect on a 2- to 4-log scale. Chitosan did not show significant antibacterial activity on its own in the absence of prior PDT, indicating that the chitosan's potentiated action only became effective after the bacterial damage brought on by PDT. Moreover, degree of PDT damage and the chitosan's degree of deacetylation seem to be connected to the chitosan's enhanced PDT action (48).

Chapter 3

Material and Methodology

The following section summarizes the methodological steps exploited to carried out the research.

3.1 Ethical Approval

The research was accepted following discussions by the Department of Life and Health Sciences at Capital University of Sciences and Technology, Islamabad, after the proposal summary was presented to the departmental Ethical review committee.

3.2 Samples Collection

Bacterial Samples were obtained from Nishtar Hospital Multan, Pakistan. The specimens were obtained from the sputum and blood of patient.To identify this bacterium species, isolates of Acinetobacter baumannii must be characterized morphologically and biochemically. The isolates were performed to the Lab at the corresponding hospital for initial identification, which involved assessing their colony/cell morphology, doing biochemical and molecular characterization, as well as performing an antibiotic susceptibility assay.

3.3 Culturing the Isolates

The Isolate was inoculated onto MacConkey agar which were used as nutrient agar to enhance bacterial growth uniformly then colonies were picked and inoculated on CHROM-agar plates, which serve as a selective medium for *A. baumannii* species. The plates were then incubated at a temperature of 37 C. Following a 24-hour incubation period, observations were made on the form, colony size, texture, and margin. In order to achieve optimal growth, colonies of *A. baumannii* were subjected to re-culturing on nutrient agar plates. The colonies were chosen from the growth plates based on their size in order to harvest DNA and carry out subsequent processing. Colonies were picked on coal swabs which were sealed properly and stored at 4C.

3.4 The Isolates' Antibiotic Susceptibility Testing (AST)

Disc diffusion method will be used for assessing antibiotic susceptibility. A bacterial overnight broth culture having turbidity of 0.5 McFarland (turbidity standard) will be inoculated on MHA medium with the help of sterile cotton swab. The antibiotic discs such as piperacillin, imipenem, meropenem, Doripenem, minocycline, levofloxacin, ciprofloxacin, Amikacine, Gentamicin, Tobramycin, Tetracycline, Tazobactam will be put to MHA medium plates and given a 24-hour incubation period at 37C.

3.5 DNA Extraction, Quantification, and Sequencing

The Solarbio DNA purification kit was employed for the purpose of DNA extraction. The quantity of DNA extracted from the isolate was determined through Qubit 2.0. fluorometer. The purity and quality were assessed via the utilization

of Agarose Gel Electrophoresis. TE buffer was used to dilute the DNA. The DNA was then sequenced on the Illumina Next Generation Sequencing 500 platform after the samples were delivered for sequencing.

3.6 Sequence Submission

After that, the sequencing data was uploaded to the National Center for Biotechnology Information Archive (NCBI) online database along with the project Accession

3.7 Sequence Retrieval

The genomes of the isolated strains were downloaded from global complete genomes were retrieved from PATRIC database based on the host (human)

3.8 Assembly and QC Check

The FastQC program (https://www.illumina.com/products/by-type/informatics-products/basespace-sequence hub/apps/fastqc.html) was used as the initial step in the genome assembly process to assess the quality of the raw sequencing data. SPAdes 3.13.0 (https://cab.spbu.ru/software/spades/) was the genome assembler used to put the raw reads together. Spades are a bioinformatic tool that has garnered considerable attention and widespread usage owing to its notable efficacy in the assembly of DNA sequences, particularly in the context of intricate genomes. SPAdes offer a precise approach for building short readings by employing a combination of de Bruijn graph and graph-based algorithms. This facilitates the ability of researchers to reconstruct genomic sequences and interpret genetic information with increased precision and enhanced continuity. The acquired data encompasses the aggregate number of contigs, genome magnitude, GC Content, and comprehensive coverage. The following parameters were determined: the total count of

contigs, the size of genome, GC content, and the overall coverage. Assembled. genomes. Were then subjected for quality check through the quality evaluation tools QUAST, developed by the Center for Algorithmic Biotechnology (CAB). QUAST (Quality Assessment) (https://cab.spbu.ru/software/quast/) utility for Genome Assemblies a bioinformatics is widely utilized for the purpose of evaluating the quality of genome assemblies. The tool offers comprehensive metrics and visualizations for evaluating the precision, comprehensiveness, and structural soundness of assembled genomes. The QUAST tool is particularly valuable for the purpose of comparing different assembly approaches and refining pipelines for the process of genome assembly. The minimum contig length option was set to zero, while the default values were utilized for the remaining parameters.

3.9   Specie Identification

After assembly and quality check genomes were run by FIDBC (http://fbac. dmicrobe.cn/tools/) to confirm the species.

3.10   Genome Annotation

The compiled genomes underwent structural annotation using the Prokka pipeline with default settings. Prokka, a bioinformatics tool designed for precise and efficient annotation of bacterial genomes, streamlines the process through gene prediction, coding region identification, and functional annotation assignment to various genomic components. Widely recognized as an indispensable tool for researchers studying microbial genomes, Prokka's automated capabilities contribute to its notable efficiency. This annotation process yielded multiple output files, including a Protein FASTA file, a Nucleotide FASTA file, and an Annotation file in GFF3 format. With the Rapid Annotation employing Subsystem Technology (RAST) server, functional annotation was carried out. RAST (Rapid Annotation utilizing Subsystem Technology) is a bioinformatics program that provides an extensive and intuitive platform for genome annotation and analysis.This tool plays a crucial

role in annotating microbial genomes, assisting researchers in uncovering genes, functional elements, and metabolic pathways embedded within DNA sequences. RAST's automated and curated methods significantly accelerate the comprehension of microbial genetics and biology. The resulting output files are available in various formats, such as GenBank and EMBL.

3.11   MLST (Multilocus Sequence Typing)

The MLST Software version 2.0.9 was utilized to analyze the contig sequences of one isolate. Renowned as a cutting-edge bioinformatics tool, MLST Software 2.0.9 is widely employed for Multi-Locus Sequence Typing (MLST) analysis. This updated version enhances precision and effectiveness in characterizing genetic diversity among microorganisms, making it an indispensable instrument for researchers and physicians investigating microbial evolution and epidemiology. The study employed the Institute Pasteur scheme (MLST-IP), utilizing seven housekeeping genes (cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB) to determine the Allelic profiles or Sequence Types (STs) of each isolate. Profile validation was conducted through PubMLST (Public Multilocus Sequence Typing), a robust bioinformatics tool for microbial strain typing and epidemiological studies. PubMLST facilitates the evaluation of genetic differences in multiple genes, enabling the monitoring and understanding of infectious disease transmission. Moreover, PubMLST fosters collaboration and data exchange within the scientific community through standardized and publicly accessible databases.RAST's automated and curated methods significantly accelerate the comprehension of microbial genetics and biology.The study employed the Institute Pasteur scheme (MLST-IP), utilizing seven housekeeping genes (cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB) to determine the Allelic profiles or Sequence Types (STs) of each isolate.a pangenome analysis was conducted on four genomes from isolated strains, along with twenty-nine draft genomes from Pakistan ,Moreover, PubMLST fosters collaboration and data exchange within the scientific community through standardized and publicly accessible databases.

3.12 Pangenome Analysis

A species' complete gene pool, comprising its dispensable and core genomes, is referred to as its pan-genome. The genes that are universally present in every genome of a specific species and necessary for bacterial proliferation make up the core genome. Dispensable sequences, on the other hand, might not be found in every genome and might support traits unique to a strain, like pathogenicity, stress tolerance, and sensitivity to different stimuli. In this study, a pangenome analysis was conducted on four genomes from isolated strains, along with twenty-nine draft genomes from Pakistan and four hundred and sixty-six complete global genomes retrieved from PATRIC, the Pathosystems Resource Integration Centre. PATRIC is a comprehensive bioinformatics framework designed for analyzing microbial genomes and associated data, allowing scientists to explore, annotate, and compare genomes, as well as conduct in-depth analyses related to microbial communities, metabolic pathways, and functional annotations. The use of PATRIC significantly advances understanding of diverse diseases and microbiomes, making it an essential tool for microorganism research. The pangenome study employed the Roary version 3.13.0 pipeline, with the input file in GFF3 format. Output file selection was based on desired results, while other parameters were kept at their default settings

3.13 Phylogenetic Analysis

The CSI Phylogeny 1.4 program is a widely employed bioinformatics tool in the realm of evolutionary research, offering exceptional capabilities for constructing phylogenetic trees and investigating genetic relationships among organisms. Featuring a user-friendly interface and advanced algorithms, this software is indispensable for scientists engaged in evolutionary biology and genetics studies. To delve into genetic variants and evolutionary links, phylogenetic relationships were established among four genomes, along with fifteen global strains and one reference genome, using CSI Phylogeny 1.4. Version 6.8 of the Interactive Tree of Life (iTOL) web software was used to visualize the generated Newick file.

(https://itol.embl.de/tree/39582304438249170395 4690.iTOL, a cutting-edge bioinformatics application, is commonly utilized for visualizing and annotating phylogenetic trees and hierarchical data. With its online accessibility, extensive customization options, and interactive features, iTOL proves essential for researchers and scientists aiming to explore and communicate intricate evolutionary relationships and taxonomy. The finalized tree was rendered and saved in PNG format.

3.14   COG Analysis

Functional annotation is the process of assigning biological functions to gene products, such as proteins or RNA molecules. It involves identifying the roles and activities of these molecules within a biological system. functional annotation using COG involves categorizing genes or proteins into orthologous groups based on shared functions, aiding researchers in understanding the biological roles of specific genes or gene products. The COG Bioinformatics tool is a highly robust and adaptable software utilized in the field of biological research. The utilization of this tool facilitates the effective examination of Clusters of Orthologous Groups (COGs), hence assisting researchers in the identification of common functional attributes across genes across diverse organisms. The COG Bioinformatics tool plays a significant role in providing useful insights into evolutionary links and gene functions through the facilitation of comparative genomics. COG analysis was performed through Bacterial Pangenome Pipeline B-Pan. B-Pan is a Bacterial pangenome pipeline which is used for pangenome, phylogenetic and COG analysis. In COG analysis Core genes and unique genes involved in different pathways were determined.

3.15 Overview of Methodology

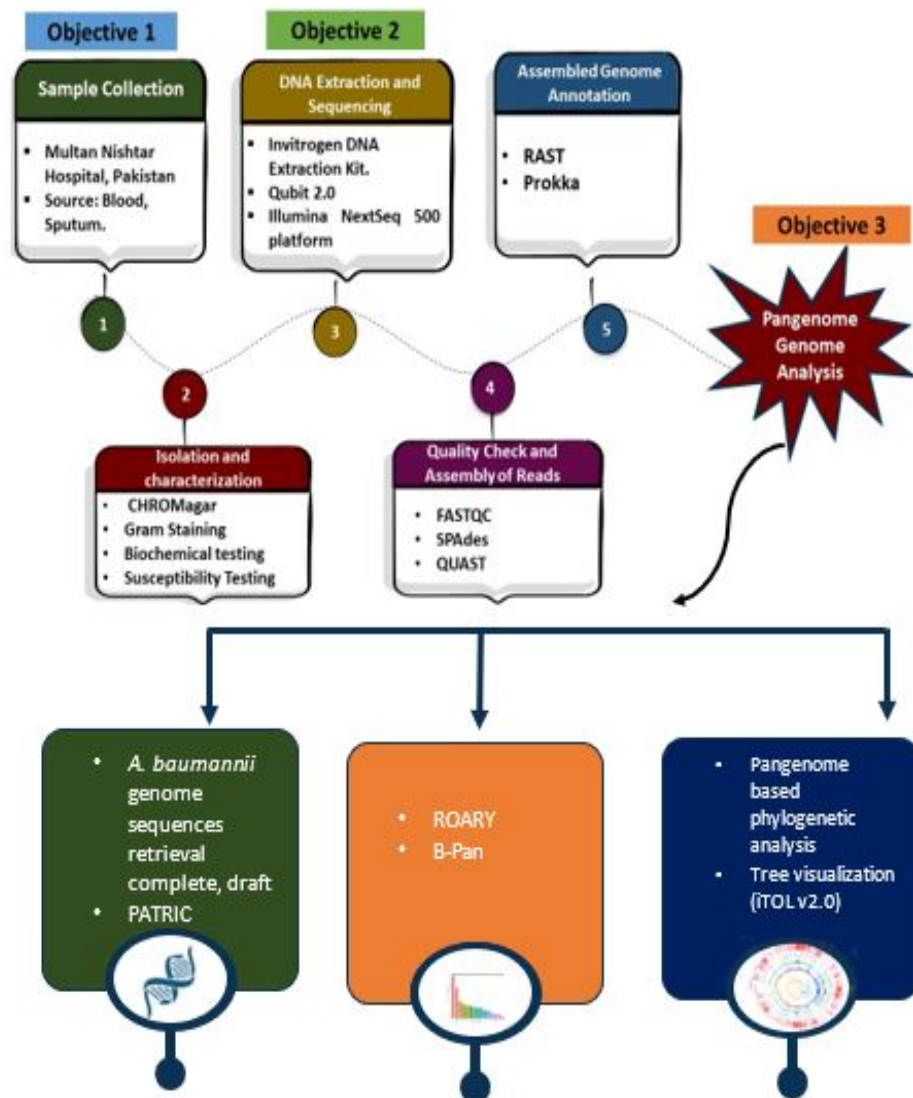The overview of methodology acquired is represented in figure 3.1

FIGURE 3.1: Methodology used for Pangenome Analysis.

Chapter 4

Results

4.1   *A. baumannii* Isolate Morphological and Biochemical Characterization

To comprehend and identify this bacterium species, isolates of Acinetobacter baumannii must be characterized morphologically and biochemically. The following are some general traits and techniques that are frequently employed for *A. baumannii*'s morphological and biochemical characterization. Morphological characterization of colony is Red on CHROMagar and Pale yellow on Nutrient agar. Its form is smooth and texture is Mucoid. Gram-negative bacteria *A. baumannii* is examined under a microscope and subjected to biochemical tests. Its oxidase test is negative, indicating that cytochrome c oxidase is absent, while its catalase test is positive, indicating that the catalase enzyme is present and that hydrogen peroxide is broken down into water and oxygen when added.

TABLE 4.1: Biochemical Characterization of *A. baumannii*..

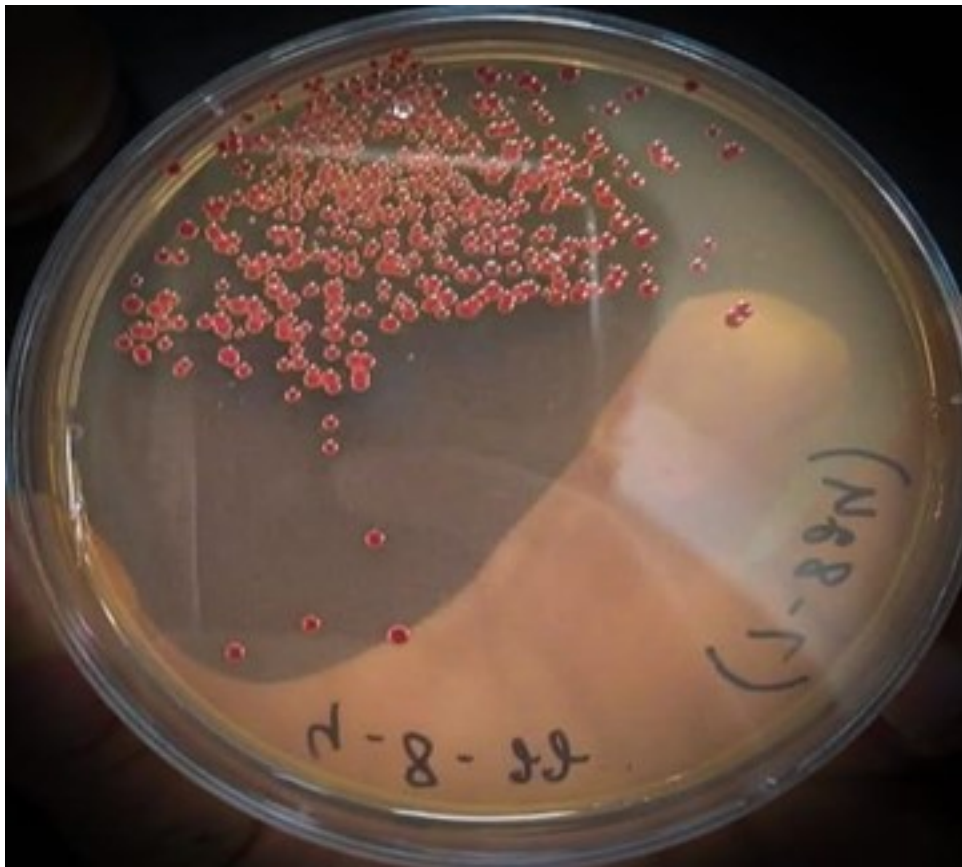| Biochemical	Characterization
Gram Staining	Gram Negative
Catalase Test	Catalase positive
Oxidase Test	Oxidase negative

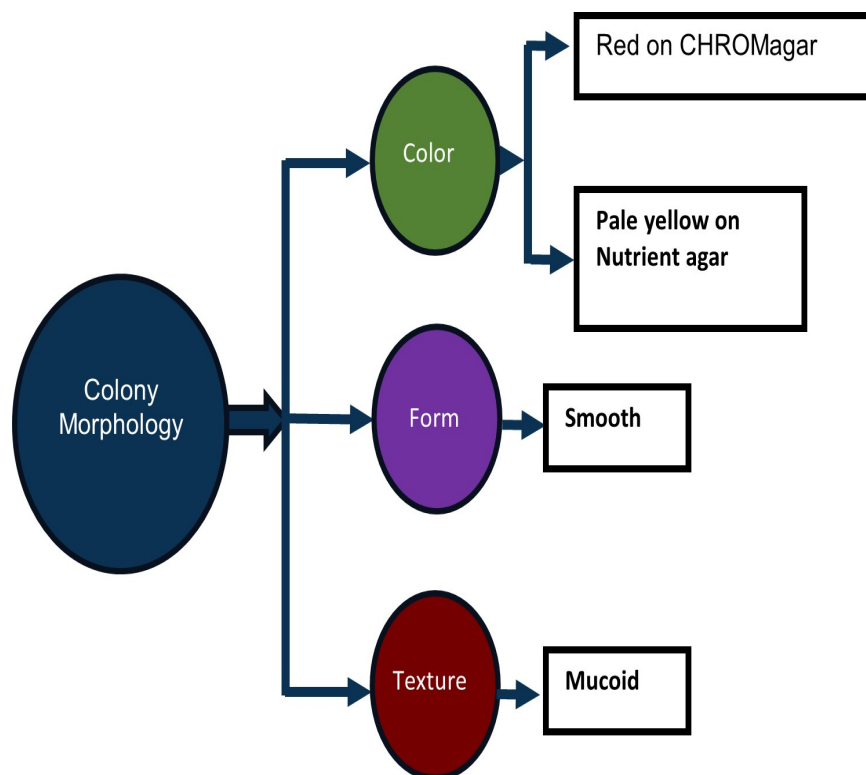FIGURE 4.1: Morphological characterization of colony

FIGURE 4.2: Flowchart of Morphological colony.

Morphological characterization of colony is Red on CHROMagar and Pale yellow on Nutrient agar. Its form is smooth and texture is Mucoid. Gram-negative bacteria *A. baumannii* is examined under a microscope and subjected to biochemical tests.

4.2 Phenotypic Antibiotic Resistance of the Isolates

The strain BAC8924 exhibited resistance to a variety of antibiotics. Specifically, it displayed resistance to the following antibiotics: Piperacillin, Imipenem, Meropenem, Doripenem, Minocycline, Levofloxacin, Ciprofloxacin, Amikacin, Gentamicin, Tobramycin, Tetracycline, Tazobactam. This antibiotic resistance profile indicates that BAC8924 is resistant to a broad spectrum of antibiotics, including beta-lactams, carbapenems, fluoroquinolones, aminoglycosides, and tetracyclines. Monitoring and understanding the antibiotic resistance patterns of bacterial strains like BAC8924 are crucial for effective clinical management and public health efforts.

TABLE 4.2: Phenotypic and genotypic antimicrobial resistance profile of local Acinetobacter baumannii strains BAC8924

Antibiotic	Disk Contents	BAC8924 PR	BAC8924 GR
Piperacillin	10μ g	R	ND
Tazobactam	$100/10\ \mu$ g	R	ND
Imipenem	$10\ \mu$ g	R	adeN, OXA-66, adeI, adeJ
Meropenem	$10\ \mu$ g	R	adeN, OXA-66, adeI, adeJ
Doripenem	$10\ \mu$ g	R	adeN, OXA-66, adeI, adeJ
Minocycline	$30\ \mu$ g	R	adeN, adeA, adeB, adeC
Levofloxacin	$5\ \mu$ g	R	rsmA, adeN, adeI, adeJ
Ciprofloxacin	$5\ \mu$ g	R	rsmA, AbaQ
Amikacin	$30\ \mu$ g	R	APH(3")-Ib, ANT(3")-IIc
Tobramycin	$10\ \mu$ g	R	APH(3")-Ib, ANT(3")-IIc

(R = resistant, S = susceptible, ND = not determined; PR = phenotypic resistance, GR = genotypic resistance)

4.3   Genomic Characteristics

De novo assembly of the Illumina data for BAC8924 produced 116 contigs longer than 500 base pairs. The overall GC content of the assembly is 38.87%. The assembly statistics include an N50 value of 88,343, N75 value of 59,298, L50 value of 11, and L75 value of 25. The longest contig in the assembly is 459,641 base pairs. The total genome size of BAC8924 is determined to be 4,017,350 base pairs. Furthermore, the annotation of the BAC8924 genome identified a total of 3,817 predicted coding sequences (CDS). Additionally, BAC8924 is found to harbor 49 tRNA genes and 3 rRNA genes within its genomic content. The circular visualization of Genomic features and characteristics of *A. baumannii* strains BAC8924.

TABLE 4.3: Characteristics of BAC8924

Characteristic	Value
Organism	*A. baumannii*
Genome size (bp)	4017350
Contigs	116
GC content %	38.87%
N50	88343
N75	59298
L50	11
L75	25
Longest contig (bp)	459641
CDS	3817
tRNA	49
rRNA	3
tmRNA	1
ST	2
NCBI Accession number	SAMN 39716321

The circuler vizulization depic the diffirent rRNA, tRNA, CDS and also have difffirent GC content.GC skews postive and GC skews negtive. This give the clear picture of genome.
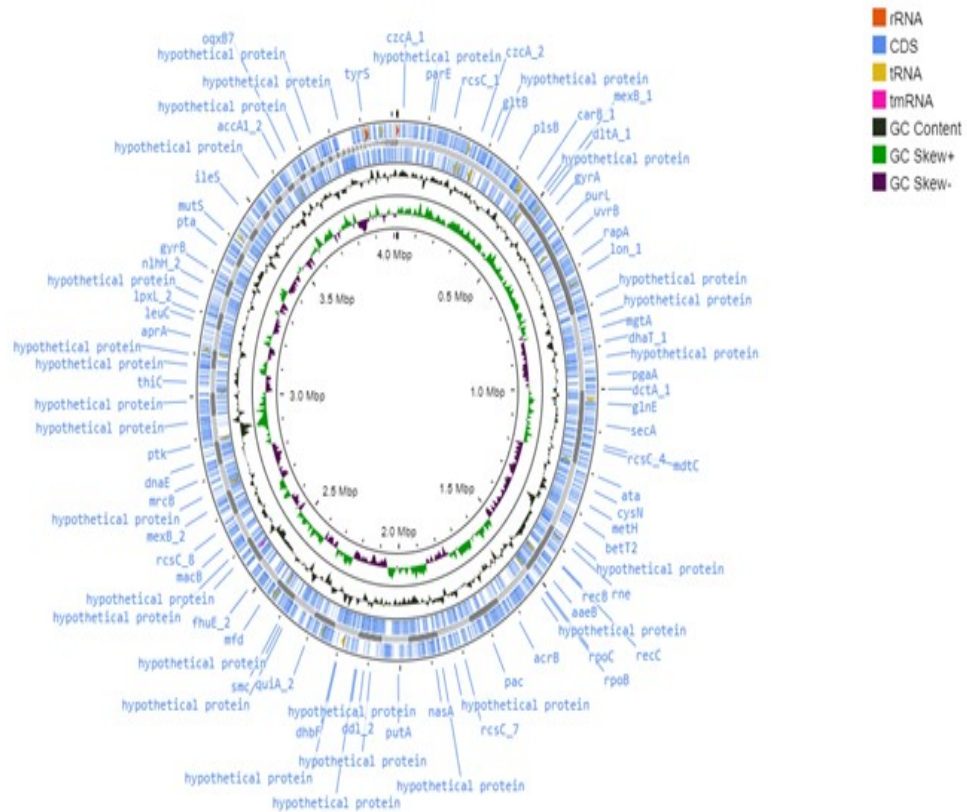
FIGURE 4.3: Circular visualization of Genomic features and characteristics of *A. baumannii* strains BAC8924

4.4 Whole Genome of BAC8924

A reference genome constitutes a thoroughly investigated and annotated genome of either the same species or a closely related one. In the context of *A. baumannii*, a suitable reference genome could be derived from a well-characterized strain. The provided figure illustrates the alignment of BC8924 with reference genomes submitted to the NCBI database. In the figure, the brown color highlights the reference genomes, while the black color represents the GC content. The purple color indicates positive GC skew, and the green color signifies negative GC skew. Additionally, blue denotes coding sequences (CDS), pink highlights assembly gaps, and parrot color is used to represent tmRNA.
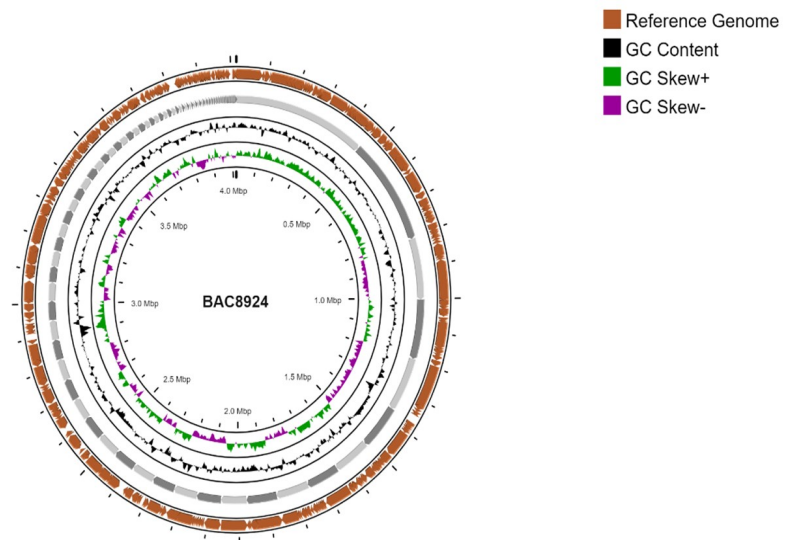
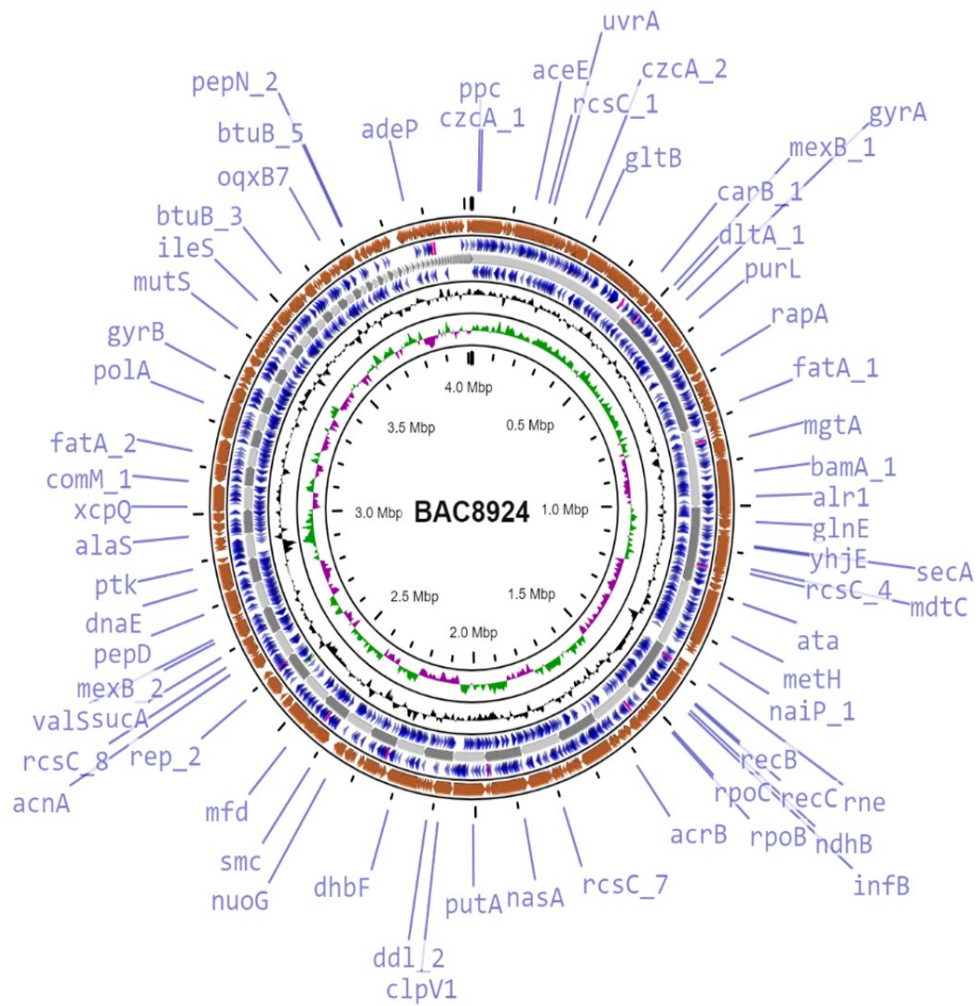FIGURE 4.4: BAC8924 with references genome

FIGURE 4.5: whole genome sequences analysis of BAC8924.

4.5 Specie Identification

Following the assembly and quality check, the genomes underwent classification using fbc.microbe.cn, which confirmed the species as Acinetobacter baumannii. The taxonomic hierarchy for this species is as follows:

TABLE 4.4: Taxonomic Hierarchy

Taxonomic Level	Classification
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Moraxellaceae
Genus	Acinetobacter
Species	baumannii

4.6 Sequence Retrieval

An extensive dataset of 196 complete genome sequences of Acinetobacter baumannii belonging to ST2 was downloaded and compiled from the PATRIC database as of December 19, 2023, all of which are from human hosts and include 28 Pakistani draft genomes that were sourced from various regions globally. Notably, all *A. baumannii* strains included in this study were characterized as multidrug-resistant (MDR) and were identified as causative agents of infections in human hosts. This collection provides a diverse and geographically representative set of genomic data for further analysis and investigation into the genetic aspects of MDR *A. baumannii* strains causing infections in different populations.

4.7 Multi Locus Sequence Typing of *A. baumannii* Isolates.

The strain BAC8924 belongs to ST2, according to the genomic analysis.All 196 *A. baumannii* samples were subjected to MLST analysis using the Pasteur scheme,

which includes seven housekeeping genes: cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB. Allelic profiles were obtained for each strain, and strains with known STs were assigned new STs. 196 strains of *A. baumannii* have significant genetic variation across their genomes, according to MLST research.The most frequent sequence type was ST2 (n=196) but none of the isolated strains belong to sequence type 2. Among other STs shared by *A. baumannii* isolates, the most frequently encountered were ST1 (84 isolates), ST437(13 isolates), ST10 (9isolates), ST57 (10isolates), ST570 (5 isolates) ST85 (8 isolates), ST78(11 isolates), ST79(6 isolates), ST622(12 isolates) ST636(13 isolates), ST40(8 isolates), ST16(8 isolates), ST422(11 isolates), ST49(14 isolates) and ST72(7 isolates).

TABLE 4.5: Housekeeping Genes, Sequence Types, and Number of Isolates

Housekeeping Genes	Sequence Types	Number of Isolates
recA, pyrG, fusA, gltA, rplB, rpoB, cpn60	ST2	269
	ST1	84
	ST437	13
	ST10	9
	ST57	10
	ST636	5
	ST85	8
	ST78	11
	ST79	6
	ST622	12
	ST636	13
	ST40	8
	ST16	8
	ST422	11
	ST49	14
	ST72	7

4.8 Pangenome Analysis with Global Strain

In the pangenome analysis conducted using Roary with a global strain set of 216 *A. baumannii* genomes, the following gene distribution was observed

- Total Genes Estimated: 19,001

- 1,326 core genes, which are conserved in 99–100% of strains

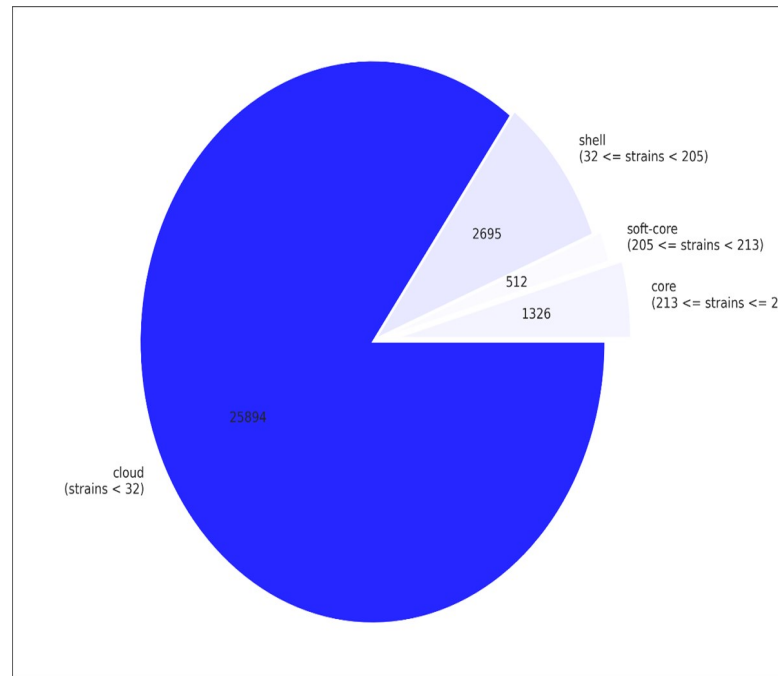- 512 Soft Core Genes (found in 95–99% of strains)

FIGURE 4.6: global strain Pie chart of gene clusters in *A. baumannii* pangenome shows Core genes, soft core genes and Accessory genes.

- 2,695 shell or accessory genes (found in 15–9% of the strains).
- 25,894 cloud or unique genes (15%of the strains)

This investigation sheds light on the genomic diversity of A baumannii by identifying a sizable pool of unique and varied accessory genes that are conserved throughout the strain collection. While the accessory and unique genes add to *A. baumannii*'s complex genomic landscape, reflecting the species' resilience and genetic variety, the core genes are highly conserved across most strains.
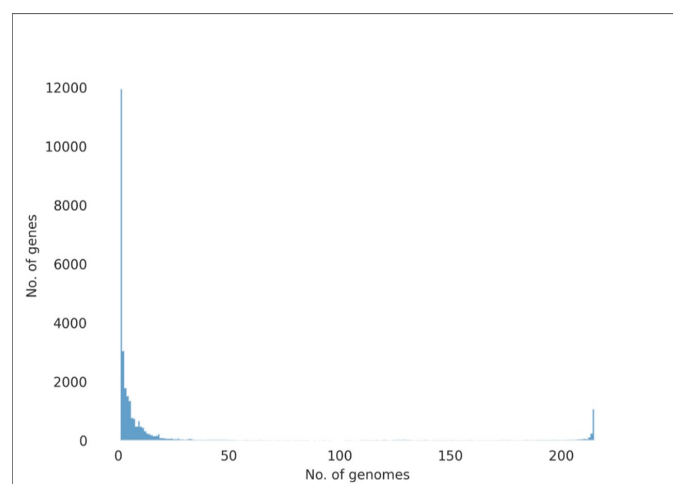

FIGURE 4.7: globally based frequency graph of genome

4.9 COG Analysis with Global Strain

The functional annotation of isolated strains shows the involvement of the Cluster of Orthologous Genes (COGs) in various functions. For strain BAC8924, 65 (19.8%) Cluster of Orthologous. Genes (COGs) are involved in translation; 47 (14.3%) in amino acid metabolism and transport; 26 (7.9%) in coenzyme
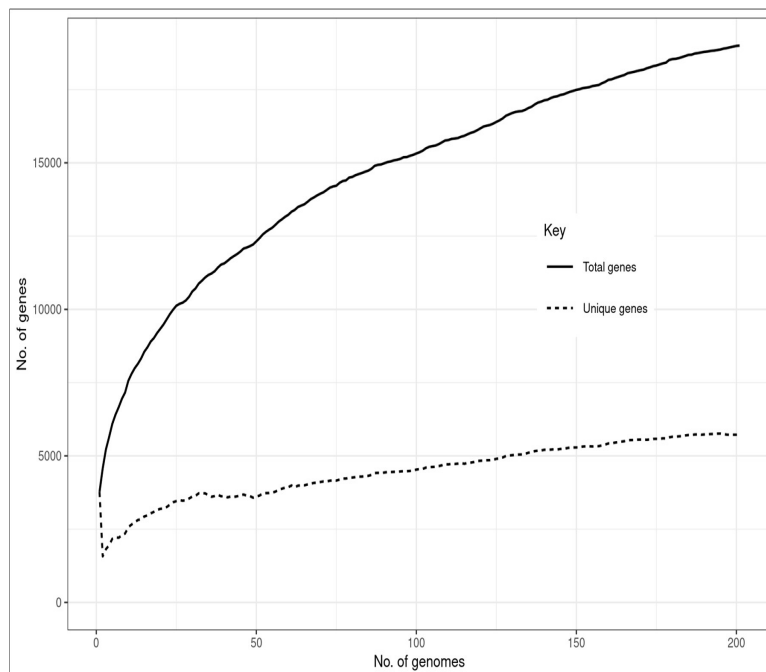

FIGURE 4.8: Graph showing increase in number of total genes with increase in number of *A. baumannii* Genomes
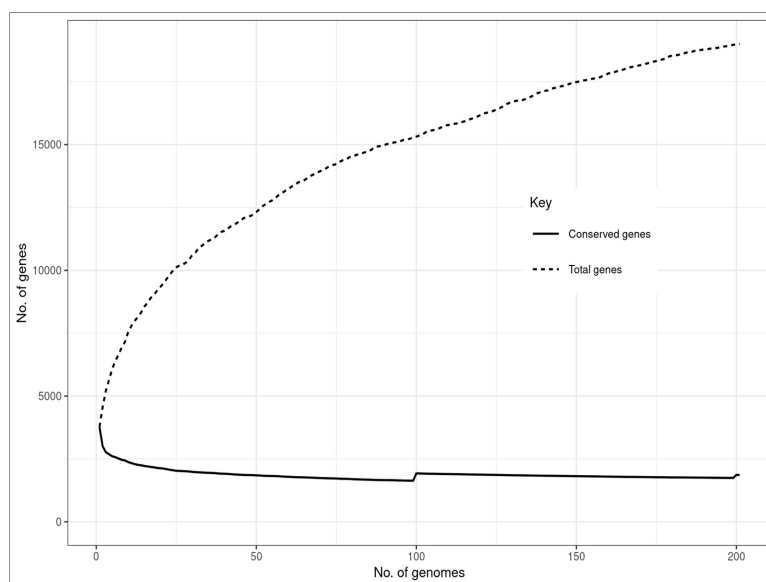

FIGURE 4.9: Graph shows increase in number of unique genes with increase in number of *A. baumannii* Genomes which indicates diversity and adoptability of isolates

metabolism; 22 (6.7%) in transcription,20(6.0%) are found to be associated with energy production and conversion; 19(5.7%) in nucleotide metabolism and transport; 17(5.1%) in General functional prediction only; 17(5.1%) in Replication and repair; 16(4.8%) in Post translational modification, protein turnover, chaperone functions; 12(3.6%) in Lipid metabolism; 12(3.6%) in Inorganic ion transport and metabolism;9(2.7%) in Carbohydrate metabolism and transport; 7(2.13%) in Cell wall/membrane/envelop biogenesis; 6(1.82%) in Secondary structure and 4(1.21%) in Intracellular trafficking and secretion. Graphical representation of the COGs involvement in various metabolic pathways are given below using the following key.

TABLE 4.6: Functional Categories, Number of Sequences, and Percentage of Sequences

| Sr. No.	Category	Functional Category	Number of Sequences	% of Sequences
0	J	Translation	65	19.82
1	E	Amino acid metabolism and transport	47	14.33
2	H	Coenzymes metabolism	26	7.93
3	K	Transcription	22	6.71
4	C	Energy production and conversion	20	6.10
5	F	Nucleotides metabolism and transport	19	5.79
6	R	General functional prediction only	17	5.18
7	L	Replication and repair	17	5.18
8	O	Post-translational modification, protein turnover, chaperone functions	16	4.88
9	I	Lipid metabolism	12	3.66
10	P	Inorganic ion transport and metabolism	12	3.66
11	G	Carbohydrate metabolism and transport	9	2.74
12	M	Cell wall/membrane/envelope biogenesis	7	2.13
13	Q	Secondary structure	6	1.83
14	U	Intracellular trafficking and secretion	4	1.22
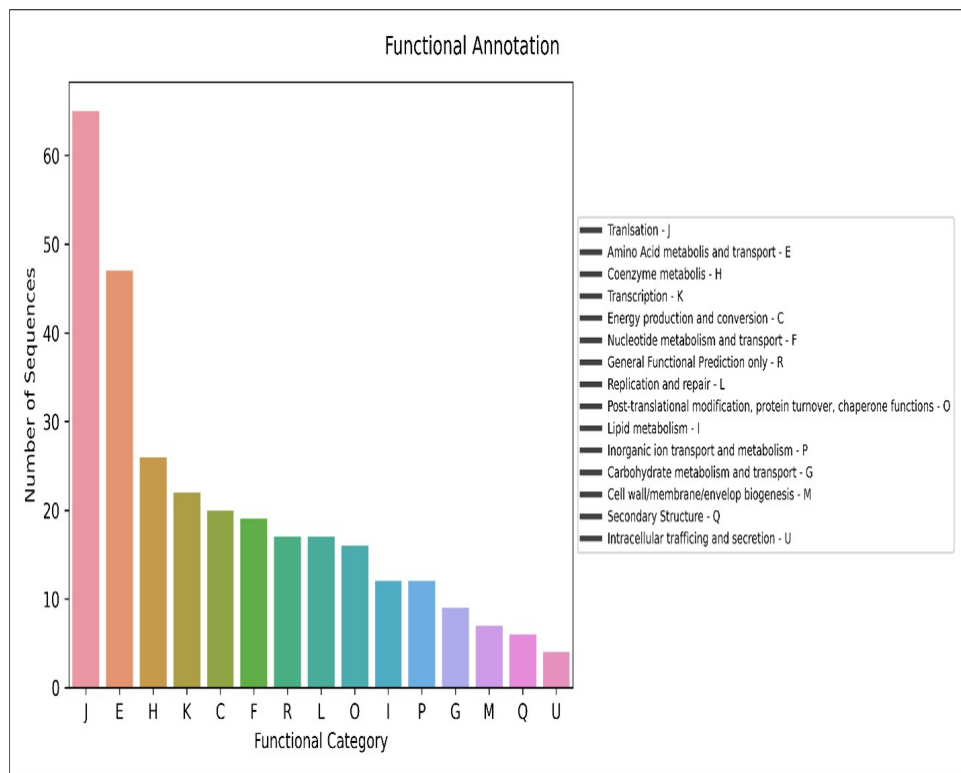
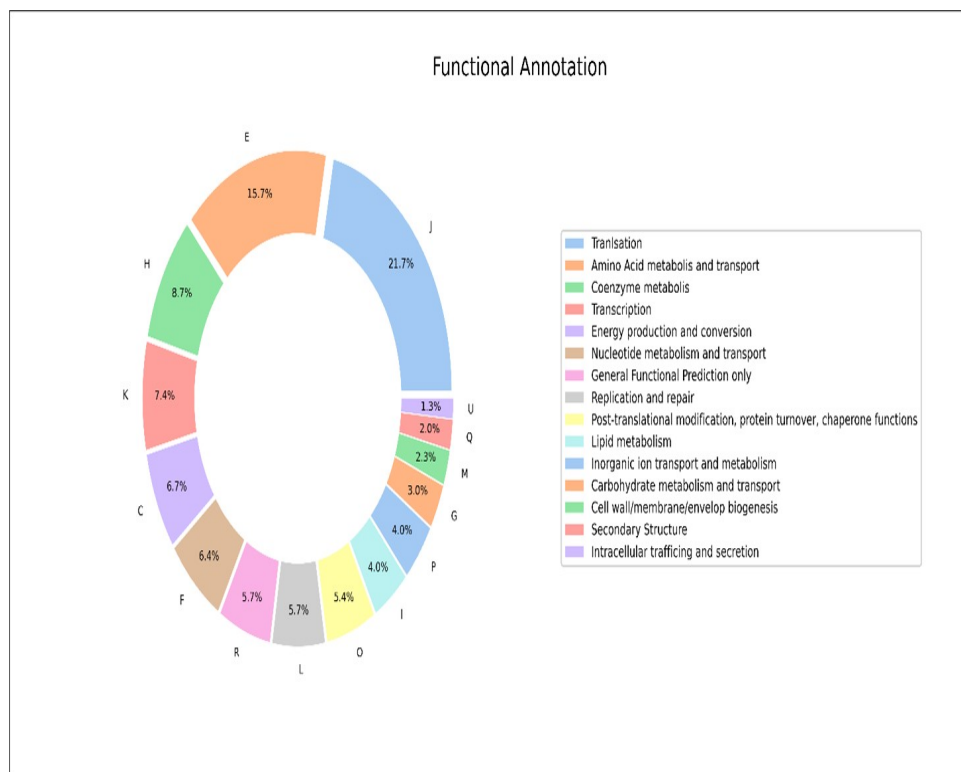FIGURE 4.10: Graph shows distribution of Cluster of orthologous genes in A. baumanni of global strains.

FIGURE 4.11: Circular visualization of Cluster of orthologous genes in A. baumanni of global strains.

4.10   Phylogenetic Analysis with Global Strain

The core-genome phylogenetic analysis of a global strain set in *A. baumannii* demonstrates that the phylogenetic relationships inferred from the core-genome align with the phylogenetic tree based on globally distributed SNPs. This consistency reinforces the reliability of the phylogenetic relationships determined using core-genome data. The phylogenetic tree was examined in further detail together with a matrix that showed which *A. baumannii* genomes included core and auxiliary genes. The findings of this analysis showed that the number of unique genes increased in tandem with the number of genomes. This discovery suggests that *A. baumannii*'s genomic repertoire extends with the incorporation of other genomes, indicating an open pangenome. The fact that the total number of genes is rising in tandem with the increasing number of *A. baumannii* genomes highlights how dynamic the genomic content of the species is. The open pangenome idea emphasizes the diversity and adaptability found in the worldwide *A. baumannii* population and represents the continuous acquisition of new genetic components
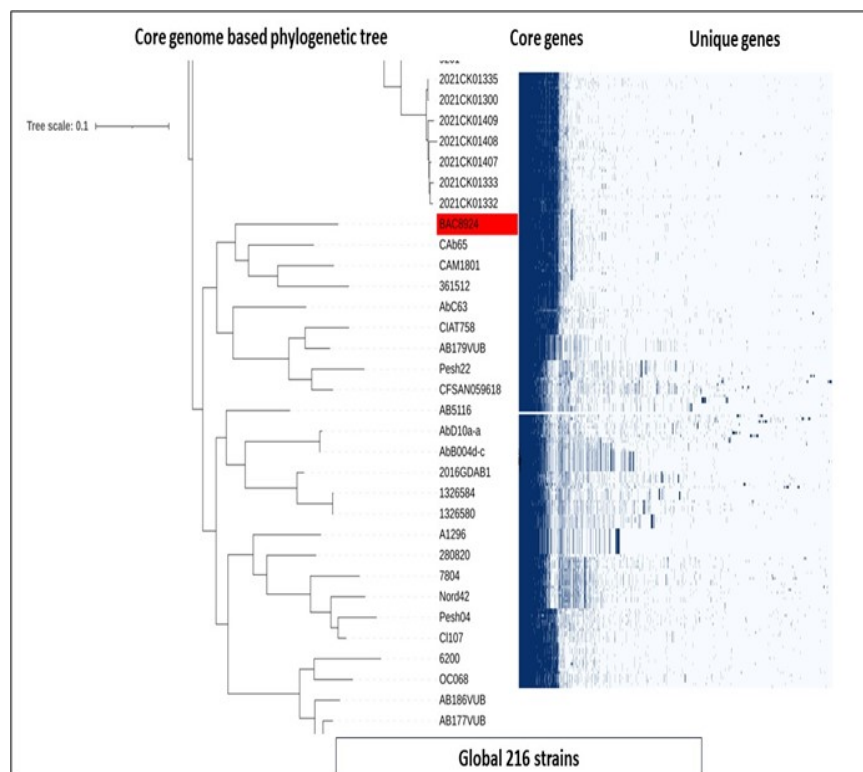

FIGURE 4.12: Pangenome tree compared to the matrix with presence/absence of core and accessory genes among *A. baumannii*

The SNP-based phylogeny revealed the presence of two main subclades. Notably, the first subclade encompassed three genomes that shared a clade, namely BAC8924, CAb65, CAM1801, and 361512. The phylogenetic analysis, which was based on the alignment of concatenated SNPs, demonstrated overall agreement with the global patterns detected in this study. The observed subclades and their composition, particularly the grouping of BAC8924, CAb65, CAM1801, and 361512 in the first subclade, were consistent with the broader phylogenetic trends identified globally.This suggests that the genetic relationships inferred from the alignment of concatenated SNPs are in accordance with the evolutionary patterns observed on a larger scale within the *A. baumannii* population under investigation.
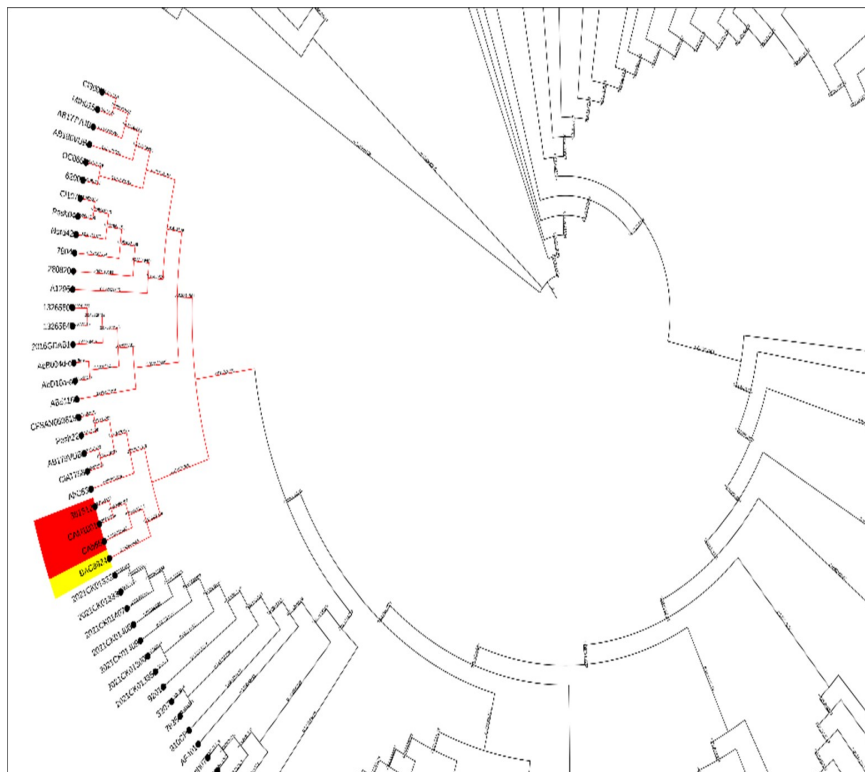

FIGURE 4.13: the grouping of BAC8924, CAb65, CAM1801, and 361512 in the first subclade, were consistent with the broader phylogenetic trends identified globally.
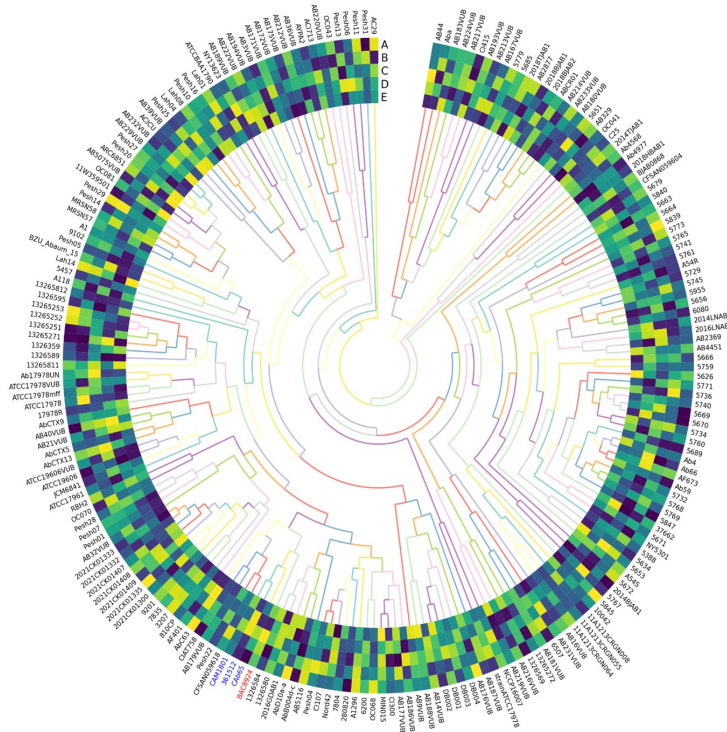
FIGURE 4.14: Circular visualization of SNP phylogenetic tree of BAC8924 global strain

4.11 ST2 Based Pangenome Analysis

The results of the ST2-based pangenome analysis provide insights into the genetic diversity and conservation within a collection of 196 strains of *A. baumannii*. Roary estimated a total of 1407 genes in 196 strains *A. baumannii* genomes, out of which 2064 were core genes these genes that are conserved among 99-100% of the strains. Core genes typically perform essential functions and are crucial for the basic biology of the organism. The fact that 2064 genes are considered core suggests a significant level of conservation across the strains. 799 soft core genes are present among 95-99% of the strains. While not as universally conserved as core genes, soft core genes still show a relatively high level of conservation within the majority of strains. 1461 were shell or accessory genes are present among 15-95 of the strains. They represent a more variable portion of the genome, with some strains having these genes and others not. This variability could contribute to differences in strain characteristics or adaptation to specific environments. 9773 cloud or unique genes that are present in only 15% of the strains. Cloud genes are

often strain-specific and might contribute to the unique characteristics or traits of individual strains.
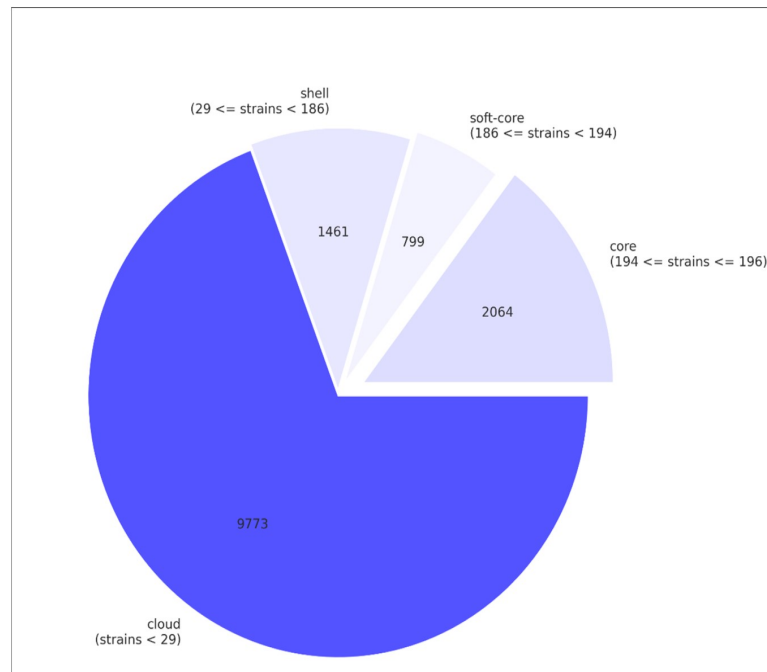

FIGURE 4.15: ST2 based Pie chart of gene clusters in *A. baumannii* pangenome shows Core genes, soft core genes and Accessory genes
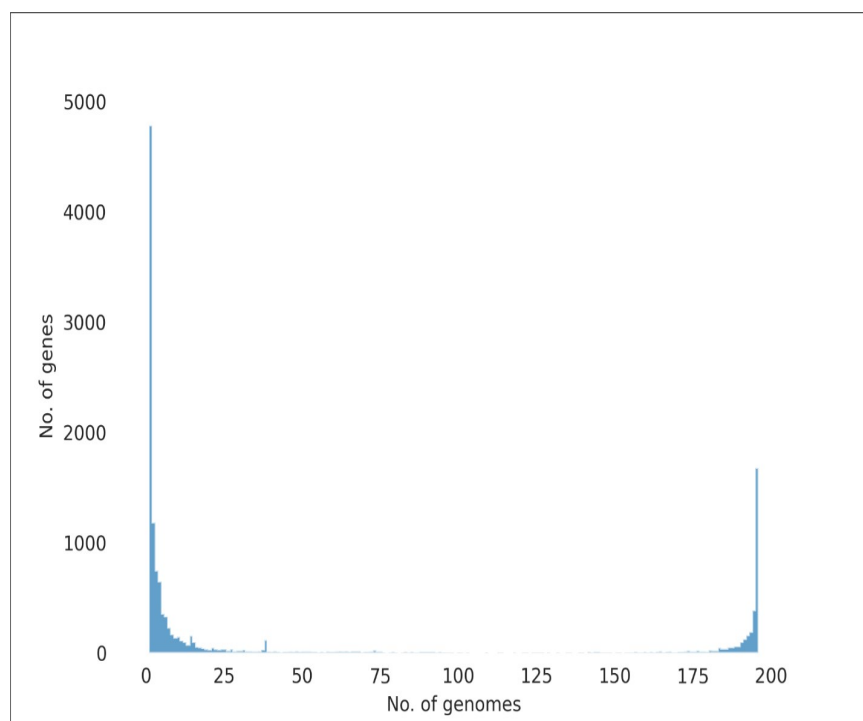
FIGURE 4.16: ST2 Based frequency graph of genome

4.12   ST2 Based Phylogenetic Analysis

The SNP-based phylogeny of Sequence Type 2 (ST2) revealed the presence of distinct subclades within BAC8924. SNP have 27 genomes. The initial subclade comprised a single genome sequence, A24, while BAC8924 shared a clade with Pesh13, Pesh11, and Pesh06. Furthermore, the phylogenetic analysis highlighted the organization of *A. baumannii* genomes belonging to ST2 into two prominent monophyletic clades. Notably, the genetic relationships deduced from the alignment of concatenated SNPs demonstrated concordance with the Sequence Types identified in this study. This consistency between the SNP-based phylogeny and other methods used for ST detection indicates a robust alignment between the genetic data and the identified Sequence Types, reinforcing the reliability and accuracy of the study's findings.

FIGURE 4.17: SNP phylogenetic tree of BAC8924 ST2 strain

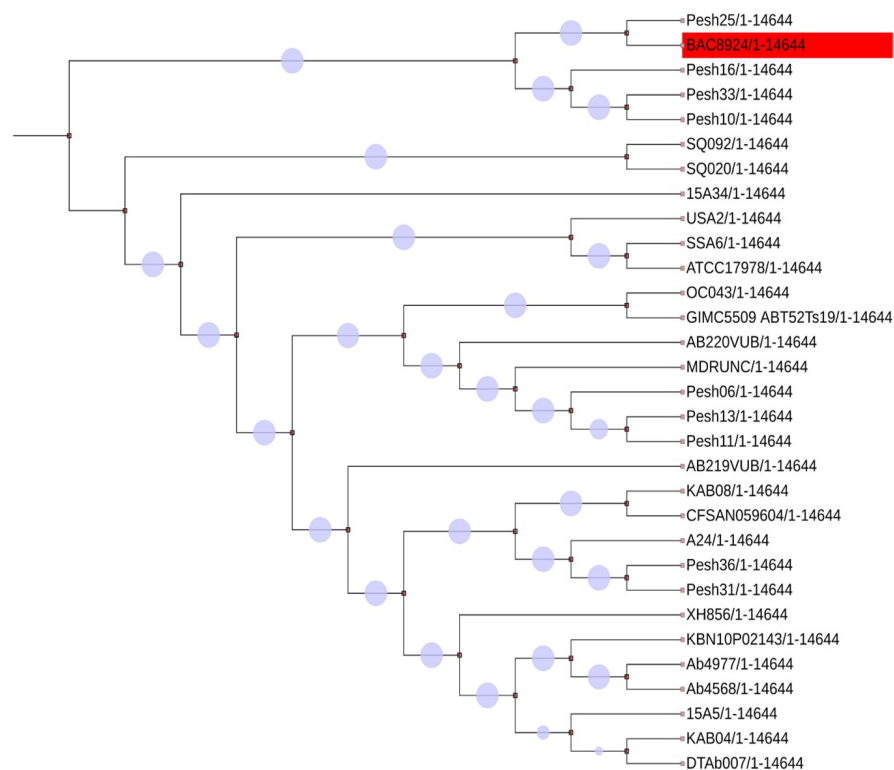In the core-genome phylogenetic analysis, the BC8924 ST2 strain clustered together with Peshawar strains Pesh13, Pesh11, and Pesh06, along with A24. This grouping in the phylogenetic tree based on the core-genome was consistent with the relationships observed in the whole-genome-based SNP phylogenetic tree (Figure 16). The strains exhibited a shared core genome of more than 80 of genes. However, the accessory genome of strain BAC8924 featured numerous genes that were not present in the majority of the other strains. The phylogenetic tree was further compared to a matrix depicting the presence and absence of both core and accessory genes among *A. baumannii* genomes. Pangenome analysis highlighted an increase in the number of unique genes with the addition of more genomes, reflecting an open pangenome. This implies that the total number of genes continues to rise as more *A. baumannii* genomes are included in the analysis. The findings suggest a dynamic and diverse genomic landscape within *A. baumannii*, with a considerable number of genes being variable among different strains.
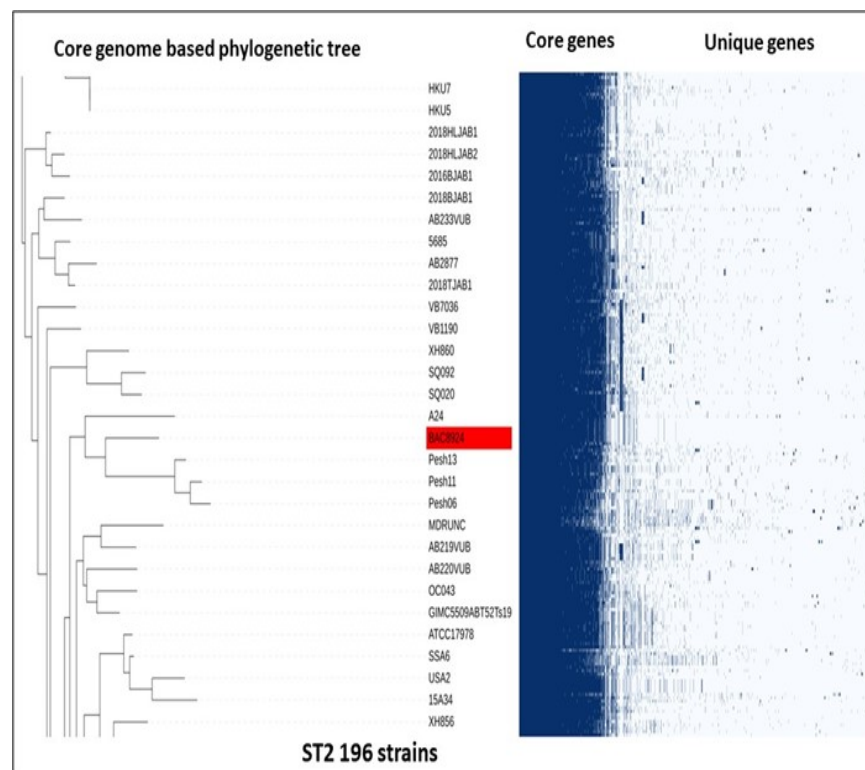
FIGURE 4.18: Pangenome tree compared to the matrix with presence/absence of core and accessory genes among *A. baumannii*.

The MLST (Multi-Locus Sequence Typing) tree, annotated with ST2 strains, including BAC8924, reveals that ST2 strains, particularly BAC8924, carry a larger

number of genes. The MLST phylogenetic tree is constructed based on varia-
tions in seven housekeeping genes: cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB.
Specifically, the ST2 196 strain, represented by BAC8924, exhibits a distinctive
genetic profile characterized by the presence of additional genes. In the MLST
phylogenetic tree, the blue-highlighted strains, notably Pesh13, Pesh11, Pesh06,
and A24, are closely related to BAC8924. These strains share a similar genetic
profile with the ST2 strains, indicating a close phylogenetic relationship. The
highlighted strains demonstrate a consistent genetic composition with ST2 strains,
further supporting their close association and evolutionary proximity to BAC8924
in the MLST analysis based on the variation in the seven housekeeping genes.

FIGURE 4.19: Circular visualization of SNP phylogenetic tree of BAC8924
ST2 strain.

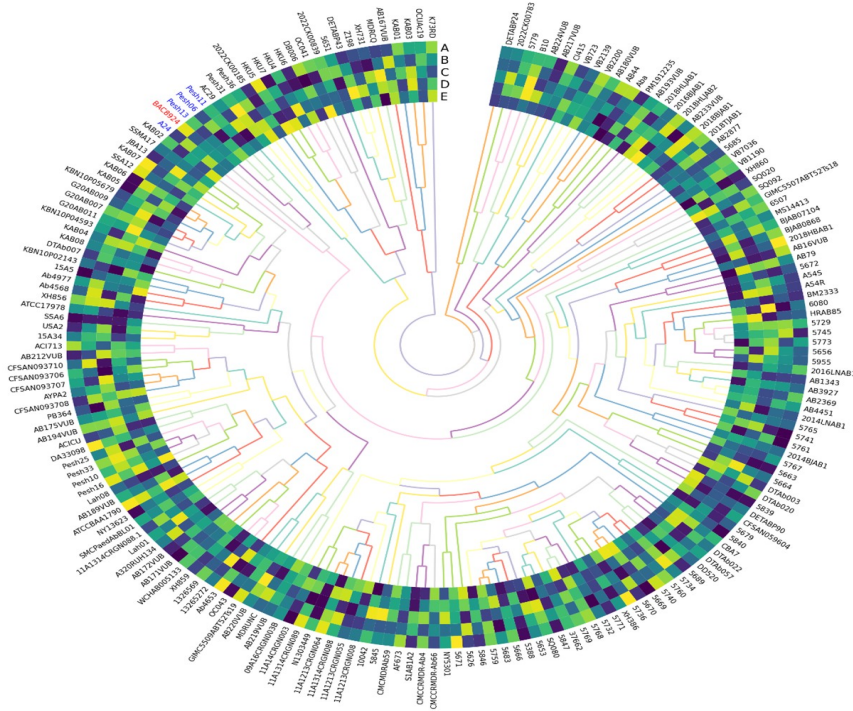# Chapter 5

Discussion

This research Identification of Genomic Diversity in *Acinetobacter baumannii* Genome via Pangenome Analysis. *A. baumannii* is a type of bacteria that is shaped like a coccobacillus and has a negative reaction to the Gram stain. It is well known for its capacity to seize opportunities and spread diseases in a variety of contexts, most notably medical ones. Because *A. baumannii* is resistant to antibiotics, it is classified as one of the ESKAPE infections. Because of this, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have identified it as a serious global health issue. This study provides valuable insights into the genetic variants and resistance mechanisms of *A. baumannii* through genome analysis. 196 strains of *A. baumannii* were analyzed using the ST2-based pangenome analysis, which sheds light on the genetic diversity and conservation within the collection. According to Roary's estimation, there are 1407 genes in all across the genomes of 196 *A. baumannii* strains; 2064 of these genes are core genes, meaning they are shared by 99–100In the pangenome analysis conducted using Roary with a global strain set of 216 *A. baumannii* genomes, the following gene distribution was observed Total Genes Estimated: 19,001, Core Genes: 1,326 (conserved among 99-100% of the strains), Soft Core Genes: 512 (found in95-99% of the strains), Shell or Accessory Genes: 2,695 (found in 15-95% of the strains), Cloud or Unique Genes: 25,894 (15% of the strains)The open pangenome of *A. baumannii* demonstrates its potential to continuously acquire genes, highlighting its genomic flexibility. The correlation between genome counts and the increase of

unique genes emphasizes the adaptability of organisms. Functional annotation by the Cluster of Orthologous Genes (COGs), provides insights on the strains' participation in diverse metabolic processes. The presence of strain-specific changes in COG involvement underscores the genetic diversity observed in functional characteristics. The variations in COG profiles offer crucial insights into the metabolic preferences of the strains, which can possibly impact their adaptation and survival strategies. The study's results highlight the pressing necessity for ongoing monitoring and investigation to proactively address *A. baumannii*'s adaptive tactics. The ST2 SNP-based phylogeny revealed the presence of two main subclades. Notably, the first subclade encompassed three genomes that shared a clade, namely BAC8924, CAb65, CAM1801, and 361512. The phylogenetic analysis, which was based on the alignment of concatenated SNPs, demonstrated overall agreement with the global patterns detected in this study. The MLST (Multi-Locus Sequence Typing) tree, annotated with ST2 strains, including BAC8924, reveals that ST2 strains, particularly BAC8924, carry a larger number of genes. The MLST phylogenetic tree is constructed based on variations in seven housekeeping genes: cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB. Specifically, the ST2 196 strain, represented by BAC8924, exhibits a distinctive genetic profile characterized by the presence of additional genes. In the MLST phylogenetic tree, the blue-highlighted strains, notably Pesh13, Pesh11, Pesh06, and A24, are closely related to BAC8924. These strains share a similar genetic profile with the ST2 strains, indicating a close phylogenetic relationship. The open pangenome nature of a genome implies a constantly changing genomic environment, which requires continuous genomic surveillance. Effective infection control strategies and antibiotic stewardship programs require collaborative efforts among researchers, doctors, and policymakers. Ultimately, this study offers a thorough comprehension of MDR *A. baumannii*, by integrating genetic studies with functional observations. The genetic diversity, antibiotic resistance, and virulence variables that have been identified provide a basis for future research and intervention efforts. This work provides valuable guidance for researchers and practitioners in tackling the growing problem of multidrug-resistant infections caused by *A. baumannii* in hospital settings. As the antibiotic resistance is increasing day by day because of the excessive use of antibiotics. Therefore, alternative strategies need to be adopted to

overcome the challenge. Vaccine development and combination therapy can play their role in combating the challenge. For better understanding of *A. baumannii* more genomes would be made available to study genetic diversity and population structure. Also, appropriate surveillance of *A. baumannii* will be needed to determine its prevalence in the region, routes of infection and local antibiotics resistance trends.

Chapter 6

Conclusion and Future Prospects

The research on *Acinetobacter baumannii*'s genomic diversity through pangenome analysis offers significant insights into the bacterial strain's adaptive strategies, antibiotic resistance mechanisms, and overall genetic makeup. The findings emphasize the continuous evolution of *A. baumannii*'s genome, indicating its ability to acquire new genes and adapt to various environments. The study's integration of genetic data with functional annotations, such as Cluster of Orthologous Genes (COGs), provides a comprehensive understanding of the bacteria's metabolic processes and functional characteristics. The SNP-based phylogeny and Multi-Locus Sequence Typing (MLST) analysis contribute to the classification and identification of distinct subclades and strains within *A. baumannii*. The study particularly highlights the genetic profile of ST2 strains, such as BAC8924, and the relationships between closely related strains in the MLST tree. Since the excessive use of antibiotics is causing an increase in antibiotic resistance every day. Therefore, alternative strategies need to be adopted to overcome the challenge. Vaccine development and combination therapy can play their role in combating the challenge. For better understanding of *A. baumannii* more genomes would be made available to study genetic diversity and population structure. Also, appropriate surveillance of *A. baumannii* will be needed to determine its prevalence in the region, routes of infection and local antibiotics resistance trends.

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