DIPARTIMENTO DI MEDICINA CLINICA E SPERIMENTALE
DOTTORATO IN BIOMEDICINA TRASLAZIONALE
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TWO STRATEGIES TO DEEPEN KNOWLEDGE THE ANTIBIOTIC RESISTANCE-RELATED TRAITS IN ITALIAN MDR STAPHYLOCOCCI: COMPARATIVE TRANSCRIPTOMIC OF TWO DAP-R/DAP-S MRSA ISOGENIC COUPLES AND CRISPR SYSTEM CHARACTERIZATION OF PATHOGENIC *S. epidermidis*

TESI DI DOTTORATO

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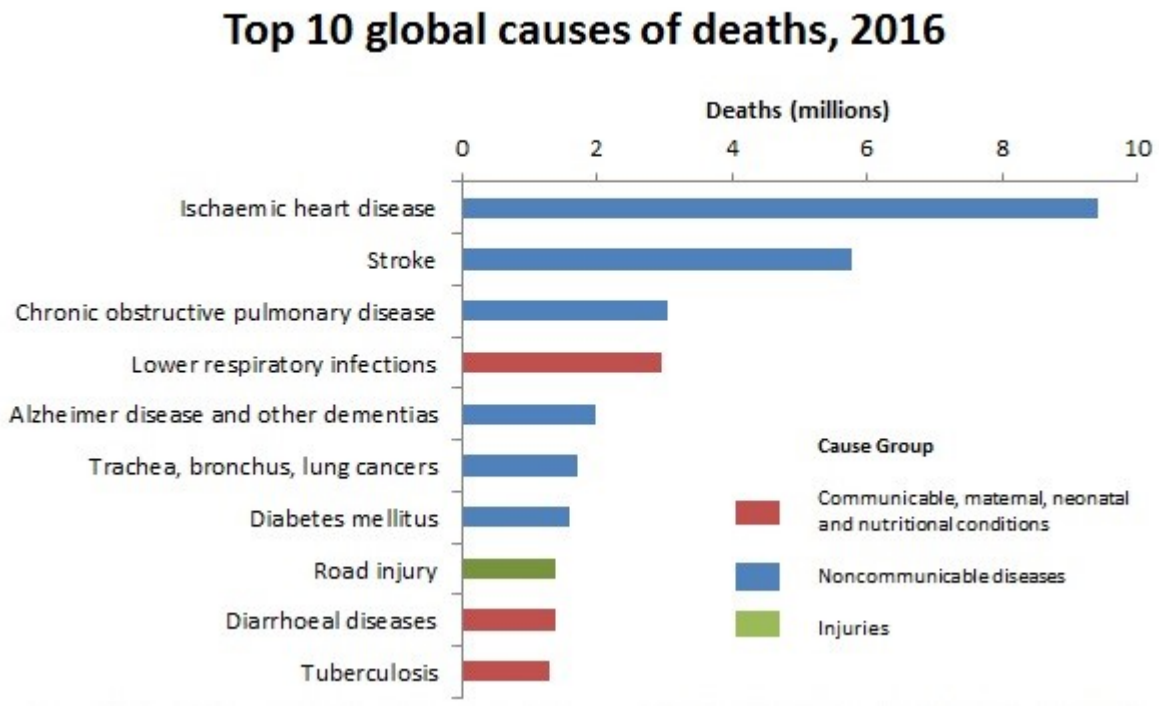
1. Introduction

1.1 The Antibiotic resistance threat

The discovery of the penicillin, the first antibiotic, by Alexander Fleming in 1928, and its introduction in clinical routine in the 1940s to cure bacterial infection, have transformed medicine, leading to the discovery of many other antibiotics, and saving millions of lives. Antibiotics significantly improved quality and average life span by changing the outcome of bacterial infections. Nonetheless, according to the World Health Organization (WHO), still in the 2016, three of the top 10 global causes of deaths were infectious diseases [1].

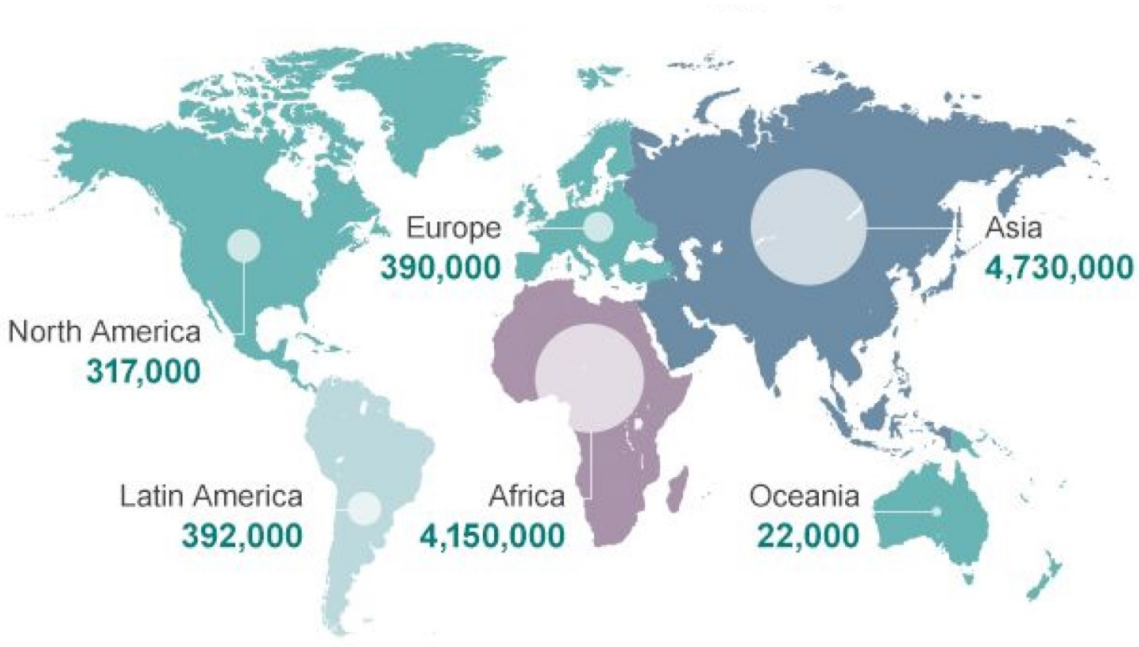


Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016, Geneva, World Health Organization; 2018

Unfortunately, as predicted by Fleming as early as 1945, the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives, led to the emerging of antibiotic resistance a few decades after the discovery of the penicillin. Antibiotic resistance was reported to occur when a

drug loses its ability to inhibit bacterial growth effectively. Bacteria become resistant and continue to multiply in the presence of therapeutic levels of the antibiotics. Often, patients do not correctly adhere to the compliance with antibiotic treatments, so the drug does not reach the optimal concentration inside the human body. Sub-inhibitory and sub-therapeutic antibiotic concentrations can promote the development of antibiotic resistance by supporting genetic, transcriptomic, and proteomic alterations. Furthermore, the inappropriate use of antibiotics kills susceptible bacteria, allowing antibiotic resistant bacteria to thrive. The incorrect antibiotics use in human medicine is not the only cause of the emerging of antibiotic resistant strains, also their massive use in agriculture and veterinary to enhance food production and reducing its cost is responsible of their lack of effectiveness, promoting the selection of antibiotic resistant strains and spreading antimicrobial agents in the environment and inside healthy organisms [2].

Antibiotic resistance is worldwide an increasing problem for the public health, indeed not only the scientific community but also the rulers of many countries proposed and implemented new plans to combat antibiotic resistant bacteria. Scientists are working hard to better know the features of these bacteria in order to develop new strategies to overcome the antibiotic resistance.



Deaths attributable to antimicrobial resistance every year by 2050 according to the WHO

1.2 Genus *Staphylococcus*

Among the most common bacteria capable to infect humans and become resistant to antibiotics, the members of the genus *Staphylococcus* are the etiological agents of some infectious diseases that can be lethal if the pharmacological treatment fails.

Staphylococcus (from the Greek: staphylē "grape" and kókkos, "granule") is a genus of Gram-positive and catalase positive bacteria; they are member of the Micrococcaceae family, are devoid of motility, and are aerobic or facultative anaerobes bacteria. Under the microscope, they appear round (cocci), and form in grape-like clusters [3]. Originally, the classification within the *Staphylococcus* genus was based on the synthesis of the pigment which gives a yellow color to the *S. aureus*, hence Staphylococci who did not have this yellow color were called *S. albicans*. In 1940, R.W. Fairbrother introduced the production of the enzyme coagulase as important principle of differentiation of species [4]. This enzyme reacts with a factor present in the plasma (Coagulase Reacting Factor, CRF), and then transform fibrinogen into fibrin in the absence of calcium ions; this protects the bacterium from phagocytosis.

Nowadays, in addition to the phylogenetic results and the different classifications that have taken place over the years, modern medicine uses a simple framework for the classification of Staphylococci based on clinical and diagnostic aspects, dividing the world of Staphylococci in coagulase-positive Staphylococci (CoPS), represented almost exclusively by *S. aureus*, and coagulase-negative Staphylococci (CoNS) [5].

Coagulase positive Staphylococci are *S. aureus* and many isolated strains of *S. intermedius* [6]. These are the most pathogenic species for humans and are involved in serious invasive or toxic syndromes. The coagulase-negative Staphylococci include *Staphylococcus auricularis*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, and *Staphylococcus simulans*. They behave as opportunistic pathogens, especially in the nosocomial infections. They can enter the body though infected wounds or catheters, and are responsible for bacteremia, urinary tract infections (especially in young women), infections on children's prostheses, and immunocompromised individuals [7].

1.2.1 *Staphylococcus aureus*

S. aureus can cause a wide spectrum of diseases, ranging from mild skin forms to systemic forms: skin infections and wounds postoperative pulmonary infections, endocarditis, meningitis, pericarditis, and food poisoning caused by the ingestion of contaminated food [8]. Approximately 30% of the human population is colonized with *S. aureus* [9]. The virulence of *S. aureus* is a multifactorial phenomenon determined by different causes involved sequentially in causing infection and disease.

S. aureus lives on the skin and mucous membranes through the factors enabling it to adhere to surfaces, and the enzymes that allow its survival. Subsequently, it penetrates the tissues using degradative enzymes that allow the spread in host tissues, and due to the production of certain factors and toxins with anti-phagocytic activities, it can evade the first natural immunity defense. Among the many extracellular substances produced by Staphylococci, some have specific activity, e.g. enterotoxins and the dermonecrotoxins, and are responsible for local or systemic pathological manifestations. During many generations, *S. aureus* has developed a wide variety of antibiotic resistance. Staphylococcal infections were treated at first with penicillin, but early these microorganisms developed a resistant phenotype, that also enabled it to survive the therapy with β-lactams of the latest generation such as methicillin (these antimicrobials typically prevent the synthesis of the bacteria's cell-wall), favoring the occurrence of so-called MRSA (Methicillin-Resistant *Staphylococcus aureus*) strains, which had a wide spread in hospitals and then in the population.

Since the 1980s, there has been a dramatic increase in the number of community-based infections due to methicillin-resistant *S. aureus* (MRSA), another example of the increasing antimicrobial resistance global trend [10].

Antibiotic resistance and biofilm-forming capacity contribute to the success of *S. aureus* as a human pathogen in both healthcare and community settings. These features are strictly related, and biofilm production of clinical isolates *S. aureus* is influenced by the acquisition of the methicillin resistance gene *mecA* [11].

S. aureus has several cell wall-associated factors that let it to attach to extracellular matrix proteins, fibrin, and platelets [12]. In particular, clumping factors A and B (ClfA and ClfB respectively) have an important role for attachment and colonization to the tissues.

Fibronectin-binding protein A (FnbA) and B (FnbB) facilitate binding to both fibrinogen and fibronectin and they are also involved in subsequent endothelial cell invasion and inflammation [13]. In addition, ClfA-B, FnbA-B, and the serine-aspartate repeat protein (SdrE) induce platelet aggregation and activation [14].

The *mecA* resistance gene in the bacterial DNA preserves the microorganisms from the action of β-lactam antibiotics, that inhibit the bacterial transpeptidase enzymes that are critical for cell-wall construction. This gene is carried on a mobile Staphylococcal Cassette Chromosome (SCC). The SCC carries a variety of genes that are responsible of the antibiotic resistance and are usually transmitted both vertically and horizontally across *S. aureus* strains. Instead of producing normal PBP-2 (Penicillin Binding-Protein 2, the enzyme responsible for catalyzing peptidoglycan cross-linking), the bacteria begin to produce PBP-2A. Non-mutated PBP-2 has an affinity for β-lactam rings whereas the PBP-2A has a low-affinity. Therefore, PBP-2A is not inhibited by the antibiotic.

1.2.2 *Staphylococcus epidermidis*

S. epidermidis is a well-known component of skin flora, as its name implies, and it colonizes the armpit, groin, anterior nares, conjunctiva, toe webs, and perineal area [15]. It is a relevant member of our microbiome and it could even have a protective function against inflammatory response through lipoteichoic acid and a mechanism involving TLR2 (toll-like receptor); it was also reported that *S. epidermidis* produces a not further characterized substance of less than 10 kD that activates TLR2, and thereby induces antimicrobial peptide production, which increased the capacity of cell lysates to inhibit growth of group A Streptococcus and *S. aureus* [16]. However, its pathogenicity has also been shown. Specifically, *S. epidermidis* has been frequently implicated in endocarditis and infections of surgical implants with reference to its capability of biofilm production as a virulence factor. *S. epidermidis* has been documented as a pathogen in skin and soft tissue infections [17]. In fact, several studies showed that *S. epidermidis* is the second most common microbe isolated from skin lesions of patients affected by atopic dermatitis. This does not directly implicate *S. epidermidis* in the pathogenesis of atopic dermatitis, due to its normal and frequent abundance on the skin, but in the most recent of those studies the

analysis of bacterial isolates from 100 patients suffering from atopic dermatitis found that *S. epidermidis* is present in the most severe skin lesions [16].

Furthermore, this bacterium is now recognized as the most frequent cause of nosocomial sepsis. The presence of CoNS in the blood (bacteremia), often originating from the dispersal of bacteria from biofilms on indwelling medical devices, can cause acute sepsis. CoNS bacteremia is associated with significant healthcare costs, morbidity, and mortality. Immune-compromised and premature neonates are the most vulnerable to CoNS sepsis with *S. epidermidis* being the most prevalent CoNS species involved [18].

Finally, *Staphylococcus epidermidis* is one of the major biofilm-producing bacteria which colonize indwelling medical devices [19]. Biofilm often contains a subpopulation of cells in a reversible non-replicative state which consequently can maintain a recalcitrant infection, these cells can be defined as being in a dormant state, generally presenting a low metabolism, allowing them to survive and resist under stressful conditions such as reduced availability of nutrients, oxygen starvation, temperature variation, salinity, pH variation, and tolerate high concentrations of bactericidal agents [20]. *S. epidermidis*, as well as *S. aureus*, can acquire methicillin resistance through the horizontal transfer of the SSC*mec*, becoming MRSE (methicillin-resistant *Staphylococcus epidermidis*). SCC*mec* types in CoNS are more heterogeneous than those in MRSA, suggesting the possibility of gene transfer in organisms other than Staphylococci, and they seem to be the source for SCCmec acquisition by *S. aureus* [21].

1.2.3 Daptomycin, Glycopeptide and Linezolid resistant Staphylococci

Daptomycin (DAP) is a fermentation product of *Streptomyces roseosporus* and it is a cyclic lipopeptide antibiotic that presents a potent bactericidal activity against most Gram-positive organisms. It is accepted for the treatment of complicated skin and skin structure infections and *S. aureus* bacteremia, including those with right-sided infective endocarditis, caused by methicillin-susceptible *S. aureus* (MSSA) and MRSA [22].

Daptomycin is also one of the few last-resort treatments for low-level vancomycin resistant (hVISA/VISA) *Staphylococcus aureus* infections.

It has a unique structure among currently available antibiotics and a new mechanism of action involving the insertion of the daptomycin lipophilic tail into the bacterial cell

membrane, in a calcium-dependent manner, causing a potassium ion efflux determining a rapid membrane depolarization. This action is followed by arrest of DNA, RNA, and protein synthesis, resulting in bacterial cell death [23].

Glycopeptides are a class of antibiotics that includes vancomycin, teicoplanin and bleomycin. Vancomycin binds the D-ala-D-ala dipeptide blocking the transpeptidase and interfering with the polymerization of the peptidoglycan, the main component of the bacterial cell-wall.

GISA (Glycopeptide-Intermediate *Staphylococcus aureus*) or VISA (Vancomycin-Intermediate *Staphylococcus aureus*) phenotypes include strains that have developed a reduced susceptibility to glycopeptides. Generally, it is known that reduced teicoplanin susceptibility can be present in *S. aureus* without a demonstrated reduction in vancomycin susceptibility [22,23], while VISA strains have demonstrated reduced teicoplanin susceptibility [24].

These phenotypes are dependent on the expression of many genes. In a consequence of repeated exposure to vancomycin, was observed a slow but gradual accumulation of mutations (35 point mutations in 31 different loci) [25,26].

In recent years, a model of the resistance mechanism of hVISA (hetero-VISA) and VISA has evolved: sequential mutation from VSSA (Vancomycin-Susceptible *Staphylococcus aureus*) lead to developing of VISA; hVISA is an intermediary between VSSA and VISA. The hetero-resistance is more difficult to detect by the usual laboratory tests and its spread was identified through the hands of healthcare workers. VISA strains are characterized by a reduced cell wall turnover, a reduced autolytic activity and, in some cases, an activated cell wall synthesis. Probably, these features lead to cell wall thickening and reduced vancomycin access [27].

Even if more rarely than GISA and VISA, glycopeptide-resistant *S. epidermidis* (GRSE) [28] and vancomycin-intermediate *S. epidermidis* (VISE) [29] strains were isolated in immunocompromised or surgery patients.

Linezolid (LZD) has become an attractive alternative to vancomycin in the treatment of infections supported by Gram-positive bacteria due to the emergence of methicillin resistance. LZD inhibits bacterial protein synthesis through binding to the peptidyltransferase center (PTC) of the 50S ribosomal subunit. Although LZD resistance

in *S. aureus* and *S. epidermidis* is still uncommon, with >99% of isolates being susceptible, to date three mechanisms responsible for LZD resistance have been reported in clinical isolates: i) mutations in the domain V region of one or more of the five or six copies of the 23S rRNA gene (e.g., G2576T, T2500A, and G2447T, *Escherichia coli* numbering system); ii) acquisition of the plasmid-mediated ribosomal methyltransferase *cfr* gene; iii) deletions or mutations in the ribosomal protein L3 of the PTC [30].

Interestingly, the *cfr* gene was first discovered in 2000 during a surveillance study for florfenicol resistance among Staphylococci from animals. It was initially detected on the 16.5-kb multi-resistance plasmid pSCFS1 from a bovine strain of *Staphylococcus sciuri* and has also been found in bovine strains of *Staphylococcus simulans*. The pSCFS1 plasmid of the bovine *S. sciuri* strain also carried the rRNA methylase gene *erm*B, the aminocyclitol phosphotransferase gene *spc*, and the ABC transporter gene *lsa*(B), which confer resistance to macrolide-lincosamide-streptogramin$_B$ (MLS_B) antibiotics, spectinomycin, and lincosamides, respectively. The *cfr* gene was recently detected on the 35.7-kb plasmid pSCFS3, from a porcine *Staphylococcus aureus* strain, together with the chloramphenicol/florfenicol exporter gene *fexA*. Cloning of the *cfr* gene and expression in *Escherichia coli* revealed that Cfr conferred resistance not only to the original Gram-positive hosts, but also to Gram-negative bacteria.

As stated before, the *cfr* gene was detected in *Staphylococcus spp*. of animal origin in Europe, but nowadays the *cfr* gene can be found in Staphyloccocci isolated from human [31] highlighting the role of the horizontal gene transfer in the spread of the antibiotic resistance. Find a method to avoid plasmid transfer and/or cure plasmid from bacterial strains could be an important step in fighting the antibiotic resistance.

1.3. Transcriptomic

Knowing every aspect about an organism genome is not necessarily enough to understand it. Genome may be constant, but some conditions can have a pronounced effect on gene expression. Although the big amount of information about the genomic traits characterizing antibiotic resistance bacteria, the understanding of bacterial transcriptomes has lagged far behind. It is well known that bacterial transcription is a biological process that is initiated from a promoter by the RNA polymerase (RNAP) holoenzyme complex, consisting of a

single RNAP core unit and a σ-factor subunit. Association with different σ-factor subunits provides promoter specificity to the RNAP core unit. In addition to the σ-factor association, transcription is either activated or repressed by a wide range of transcription factors [32]. The transcriptome gives us much more information about a functional readout of the environmental effects, so analysing it, in a specific development stage or physiological condition, is essential for interpreting the elements of a genome. Transcriptome is the complete set of RNA (mRNA, rRNA, tRNA) as well as other non-coding RNAs transcribed from the genome under specific conditions, in a specific cell, in a clearly defined time or development stage.

The development of new high-throughput sequencing technologies has revealed many unexpected features in the bacterial transcriptome, including gene structures, multiple promoters, and RNA-based regulation. These new findings suggest that bacterial transcription is much more complicated and subtle than previously thought. [33]

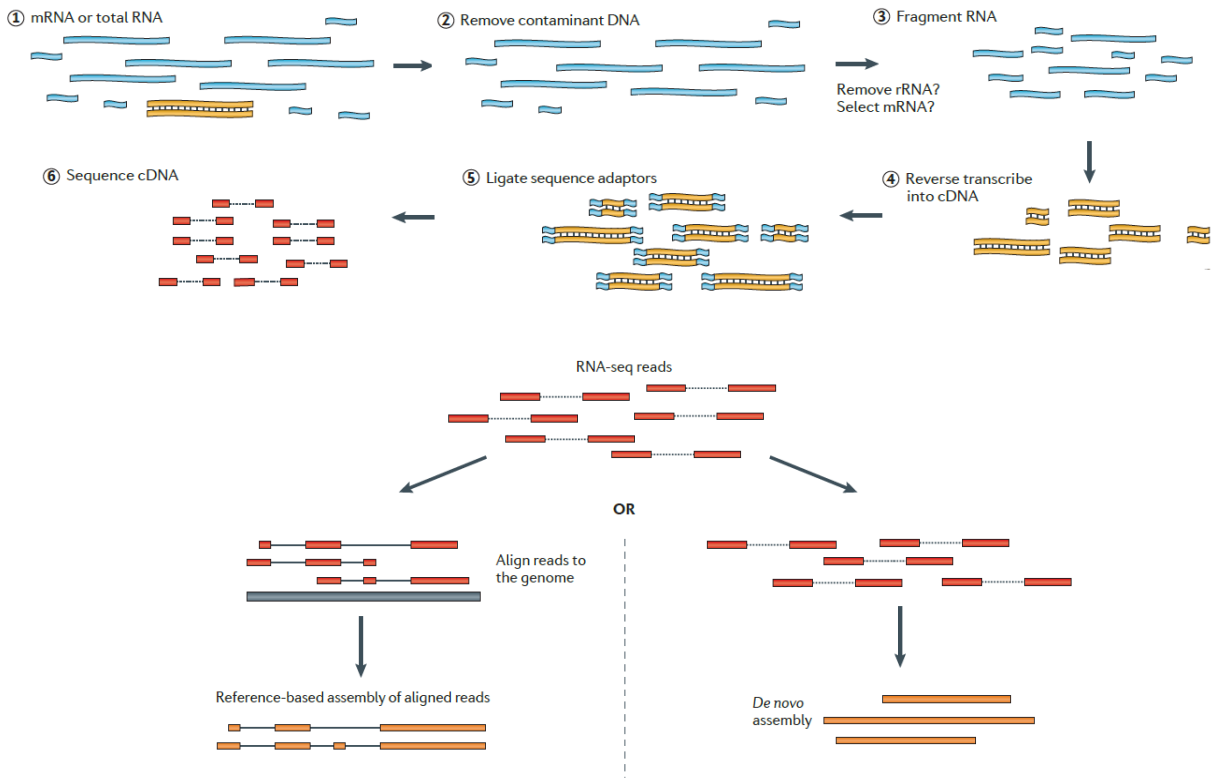
1.3.1 RNA-seq

RNA-sequencing (RNA-seq) uses the Next Generation Sequencing (NGS) technology to investigate the RNAs in a biological sample [34,35] analyzing the cellular transcriptome. Through the use of RNA-seq, it is possible to examine the alternative gene spliced transcripts, the post-transcriptional modifications, gene fusion, and changes in gene expression [36].

In addition to mRNA transcripts, RNA-seq can also study small-RNA, miRNA, tRNA, and ribosomal profiling [37]. However, bacterial mRNA can exist as little as 1% to 5% of total RNA, so mRNA enrichment is a challenging step to obtain sufficient transcript coverage. Currently, several methods are being used to remove the rRNA and tRNA fraction from the total RNA pool. Among those methods, terminator 5'-phosphate-dependent exonuclease treatment has been successfully applied to enrich primary transcriptome by reducing processed or degraded RNAs with a 5'-monophosphate end (e.g., rRNAs and tRNAs). To remove the rRNA fraction from the total RNA pool, Ribo-Zero rRNA removal kits, using biotinylated probes that selectively bind rRNA, have been used successfully for a wide range of organisms, from bacteria to human. A population of RNA is reverse transcribed to a library of cDNA fragments with adaptors attached to one or both ends. All molecule, with

or without amplification, is then sequenced to obtain short sequences from one end (single-end sequencing) or both ends (paired-end sequencing). Usually, the reads are 30–400 bp, according to the DNA-sequencing technology used. Theoretically, every high-throughput sequencing technology [38] can be used for RNA-seq: the Illumina IG18 [39-41], Applied Biosystems SOLiD and Roche 454 Life Science [42-44] systems have already been used for this purpose. After sequencing, the resulting reads are aligned to a reference genome or reference transcripts or assembled de novo to produce a genome-scale transcription map. The last contains information of both the transcriptional structure and/or expression level of each gene.

RNA-seq is still a technology under active development, but it offers different advantages over existing technologies. First, differently from hybridization-based approaches, RNA-seq is not limited to detecting transcripts that correspond to known genomic sequence. A second advantage of RNA-seq compared to DNA microarrays is that RNA-seq has very low, if it exists, background signal because DNA sequences can be unequivocally mapped to unique regions of the genome. RNA-seq does not have an upper quantification limit which correlates with the number of sequences acquired. Therefore, it has a wide dynamic range of expression levels over which transcripts can be detected. RNA-seq is highly accurate to quantify the expression levels, as demonstrated using quantitative PCR (qPCR) [45] and spike-in RNA controls of known concentration [46]. The results of RNA-seq also show high levels of reproducibility for technical and biological replicates [47]. Finally, because there are neither cloning steps nor amplification step, RNA-seq requires low levels of RNA sample. Thanks to these advantages, RNA-seq is the first sequencing-based method that allows the entire transcriptome to be analyzed in a very high-throughput and quantitative way. Therefore, a single experiment can provide information normally obtained with more essays at a much lower cost.



Data generation steps of a typical RNA-seq experiments and alternative approaches for transcriptome assembly – modified from Martin *et al.* [48]

1.3.2 small-RNA

In recent years, different sequencing-based studies have further demonstrated the increased transcriptional complexity within the bacterial genome structure. The recent discovery of small regulatory RNAs (sRNAs) in bacteria, a heterogeneous group of molecules that modulating different pathways related to diverse physiological responses, can help scientists in answering different questions related to the regulation but also can open new path to study new targets and new diagnostic tools [49-56]. Many of these works have shown that sRNAs might modulate virulence gene expression, cellular differentiation, metabolic functions, adaptation to environmental conditions and pathogenesis.

Regulatory RNAs in bacteria are usually not translated and comprise a size range between 25 and 400 nucleotides in length. They can modulate transcription, translation, mRNA stability, and DNA maintenance or silencing. These functions are achieved through a variety of mechanisms, including changes in RNA conformation, protein binding, base pairing with other RNAs, and interactions with DNA [57,58].

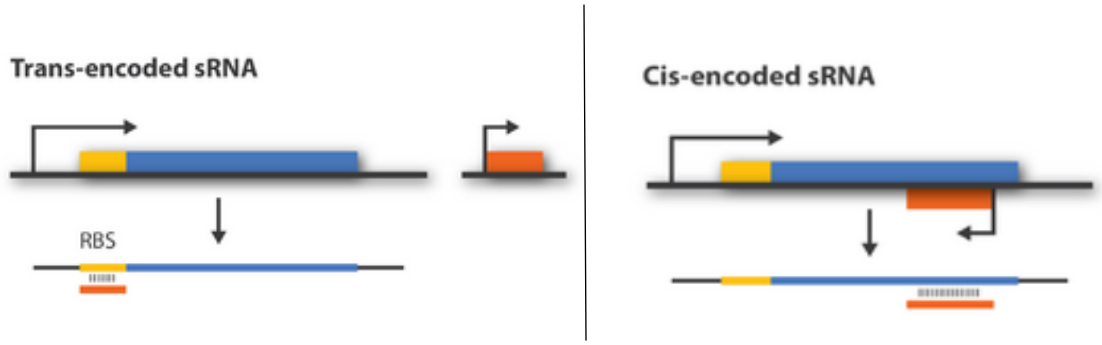
A first class of RNA regulators includes Riboswitches, which bind directly to a target molecule regulating the expression of a gene. The riboswitches are present in the 5' untranslated regions of the same mRNA that they regulate, then the mRNA is directly involved in the regulation of their activities [59]. Riboswitches are divided into two parts: an aptamer and an expression platform. The aptamer directly binds the small molecule and the expression platform show structural changes in response to the changes in the aptamer. The expression platform is what regulates gene expression. They regulate gene expression through pre-mature transcription termination or repression of translation initiation.

As already mentioned, there are two different classes of sRNA:

- Trans-acting sRNAs present on the chromosome in a location distinct from their target and show only limited complementarity with their targets, so many trans-encoded RNA molecules engage the RNA chaperone protein Hfq [60]. The general mechanism of trans-acting sRNAs is to sequester the ribosome-binding site (RBS) of a target mRNA by base-pairing to the Shine–Dalgarno (SD) sequence or the start codon and may also interact with the coding sequence of the mRNAs [61]. Most of trans-sRNAs are coupled with the activity of RNases, which are enzymes involved in RNA turnover through RNA cleavage [62-64];
- Cis-encoded antisense RNAs are complementary to their target RNA as they are transcribed from the DNA strand opposite to the genes that they regulate, so they can interact autonomously. They are often located in the untranslated regions (UTRs) of the corresponding gene where the RNA duplex formation can affect ribosome-binding/translation, termination events or overall stability of the mRNA by rearranging the secondary structures, such as hairpins in the target RNA [65].

These regulatory RNAs can act by binding to proteins and modulating their activity, whilst the majority of characterized sRNAs act by base pairing with target mRNAs. However, their functions and mechanisms are still unexplored. For the few sRNAs with known functions, some are involved in the bacterial density detection, in modify cell surface properties for host immune escape, adjust central metabolism for optimal growth, regulate the expression of virulence factors, influence antibiotic resistance, trigger cell death, and in toxins encoding [66,67].

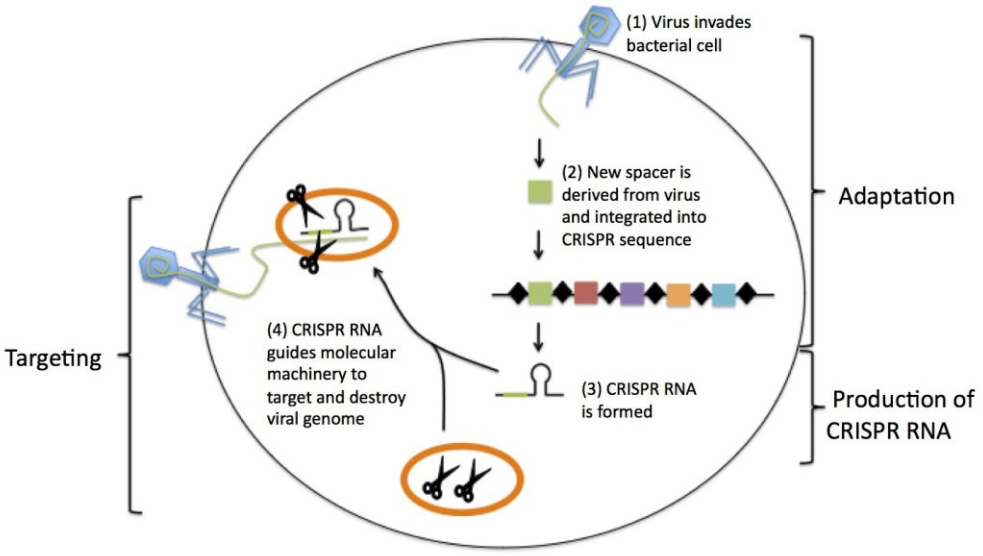
In addition to regulatory sRNAs, there is another type of sRNAs produced as part of the bacterial immune system named CRISPR.



Orange boxes illustrate the sRNA, blue boxes the target mRNA and yellow boxes the ribosome binding site

1.4 CRISPR-Cas system

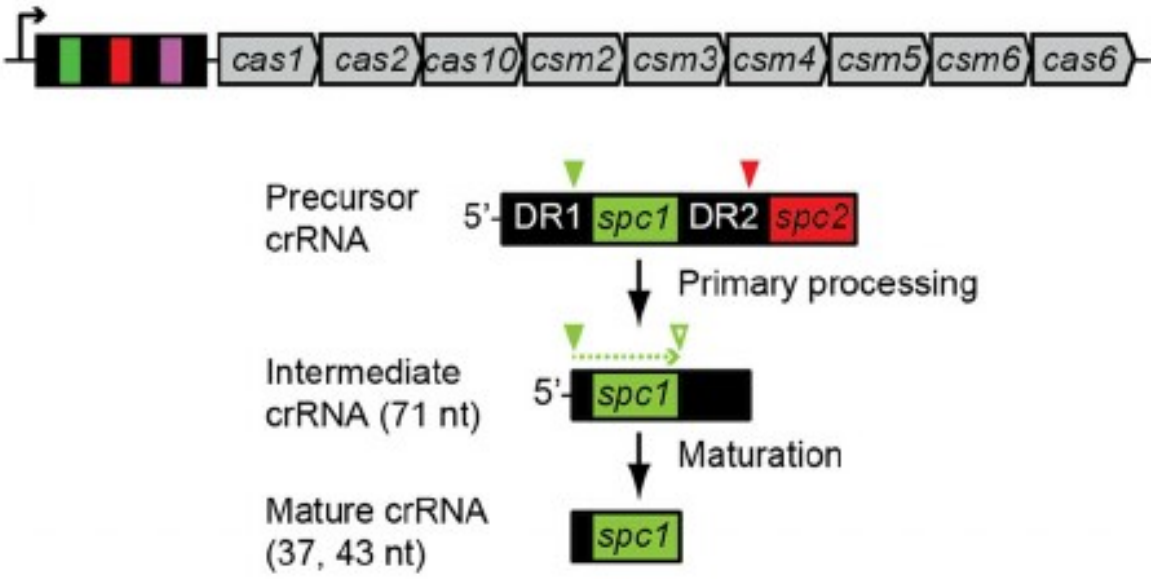
Widespread in bacteria and archaea, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR-associated (*cas*) genes constitute an adaptive immune system that uses small-RNAs (crRNAs) to protect against virus infection and plasmid transfer [68,69]. A CRISPR locus consists of an array of short repeat sequences (24 to 48 nucleotides [nt] in length) that alternate with similarly sized invader-derived sequences called spacers [70]. CRISPR loci are often flanked by an operon of *cas* genes, which encode the machinery for CRISPR immunity. There are three distinct phases of this immune system. During the first phase, known as adaptation, viral or plasmid challenge stimulates the addition of spacers to the archive of invader-derived sequences in the CRISPR locus. In the second phase, known as crRNA biogenesis, the repeat-spacer array is transcribed into a long precursor, which is subsequently chopped to generate mature crRNAs that consist of a single spacer flanked by partial repeat sequences on one or both sides. In the third phase, known as targeting, the crRNAs assemble with Cas proteins to form a surveillance complex that recognizes and destroys foreign genetic elements antisense to the crRNAs.



Phases of the CRISPR immunity

More than 12 distinct CRISPR-Cas systems have been documented, and a recent comparative analysis has led to their classification into four types based upon their architecture, *cas* gene content, and mechanisms of action. Staphylococci commonly have the type III-A CRISPR-Cas system, well characterized in the *Staphylococcus epidermidis* RP62a, an opportunistic pathogen. This system contains nine *cas-csm* genes that can direct anti-plasmid and anti-phage immunity. In this system, crRNAs are generated from the precursor transcript in two steps: primary processing and maturation. During primary processing, endoribonucleolytic cleavage events within repeat sequences liberate intermediate crRNAs that are 71 nt in length. During maturation, these intermediates are trimmed on the 3′ end in 6-nucleotide increments to form a collection of mature crRNAs that range from 31 to 67 nt in length.

The nine *cas-csm* genes of the type III-A CRISPR-*cas* system are: *cas*1, *cas*2, *cas*10 (also known as *csm*1), *csm*2, *csm*3, *csm*4, *csm*5, *csm*6 and *cas*6. *csm*2, *csm*3, and *csm*5 gene products form a stable ribonucleoprotein complex, along with Cas10 and Csm4, named Cas10-Csm complex, responsible for the crRNA maturation and DNA cleavage [71,72]. Two different sets of repeat-spacers (R/S) sequences flank the genes, one before *cas*1 (upstream of the *cas* locus) and one after *cas*6 (downstream of *cas* genes).



S. epidermidis RP62a type III-A CRISPR-Cas locus and crRNA maturation

Several papers addressed the role of the CRISPR-*cas* genes and their products:

- Cas1 and Cas2 are responsible for the adaptation [73];
- Cas10 is a nuclease that cuts the DNA [72];
- Csm2 forms dimers [74], it has a role in the crRNA maturation [71], and other unknown functions still in study;
- Csm3 is the backbone of the Cas10-Csm complex [75], it measures the extent of crRNA maturation [71], and cuts target RNA through HD domain [72];
- Csm4 has unknown functions;
- Csm5 is predicted to be a nuclease [76], it has a role in the maturation of the crRNA [75], and it recruits cellular ribonucleases [77].
- Csm6 seems to be essential for anti-plasmid CRISPR immunity, and it should act as a transcriptomic factor [76];
- Cas6 is also essential for immunity function [78], due to its nuclease activity it plays a role in primary processing of the crRNA [71].

Some CRISPR systems might be involved in gene regulation as suggested by the existence of self-targeting spacers in CRISPR arrays. The presence of such spacers is surprising considering that self-targeting is known to lead to cell death, however the presence of such spacers can be explained by the mutation or deletion of the *cas* genes and they are strongly over-represented among the first spacers of the array, indicating that their acquisition was recent. Together, those facts strongly suggest that the acquisition of a self-targeting spacer is detrimental, leading to autoimmunity, and selects for the loss of CRISPR function [79]. The same authors highlighted the regulatory function of crRNAs, making them part of a fine regulation mechanism.

Isolated *cas* genes are also involved in DNA repair, as found in the type I Crispr-Cas system [80].

The presence of CRISPR-Cas systems in multi-drug resistant pathogenic strains is an important trait to evaluate, because it can decrease the spread of plasmids carrying antibiotic resistance genes, but at the same time it appears to be related to genome plasticity [81], and it provides resistance also to phage infection, representing a trouble in the development of a phage therapy.

1.5 Phage therapy

Bacteriophages (phages) are viruses able to infect bacteria and only them. They were discovered in the early 1900s by Ernest Hankin, Nikolay Gamaleya, Frederick Twort and Felix d'Herelle. The last was the scientist who isolated these agents from the stool samples of dysentery patients, named them bacteriophages, and developed the phage assays which remain in use up to the present. This found gave the input to start a new anti-bacterial therapy based on the phage ability of kill their hosts. Less than ten years after the first phage therapy experiment, Fleming discovered the penicillin starting the antibiotic era. Due to the broad spectrum of action of these drugs and the relatively poor understanding of phage biology, antibiotics become the principal, if not the only, treatment for bacterial infections. [82]

Nowadays, the use of phages or their derivatives to treat bacterial infections has re-gained attention due to the decrease effectiveness of antibiotics and the better understanding of phage biology, phage-bacteria interaction, and the basis for bacterial infections [83]. The

FDA (Food and Drug Administration) already approved the use of a phage preparation as an antimicrobial agent to preserve ready-to-eat meat and poultry products against *Listeria monocytogenes* infection [84], moreover several research groups are working on phage-based therapy with different goals, e.g. to reduce antibiotic use in foods, as in the infant milk formula powders [85], and to cure *S. aureus* infections [86].

Phages are found in almost all environments on Earth, ranging from soil, sediments, water (both river and seawater), and in/on living or dead plants/animals. Phages can be isolated from almost any material that will support bacterial growth. The estimated global phage population size is extraordinarily high, it is estimated that only the aquatic environments have a total phage population above 10^{31} [87,88]. Phages have evolved an array of shapes, sizes, capsid symmetries, and structures. All are composed of a nucleic acid, representing the genome that can be double- or single-stranded DNA or double- or single-stranded RNA, encapsulated by a protein coat (capsid). Capsids exist in different forms, ranging from small 3D hexagon-like structures to filaments to highly complex structures consisting of a head and a tail. From a morphological perspective, 96.3% of studied phages has a tailed morphology. To date, phages are classified into fourteen families based on morphology and genome characteristics [83].

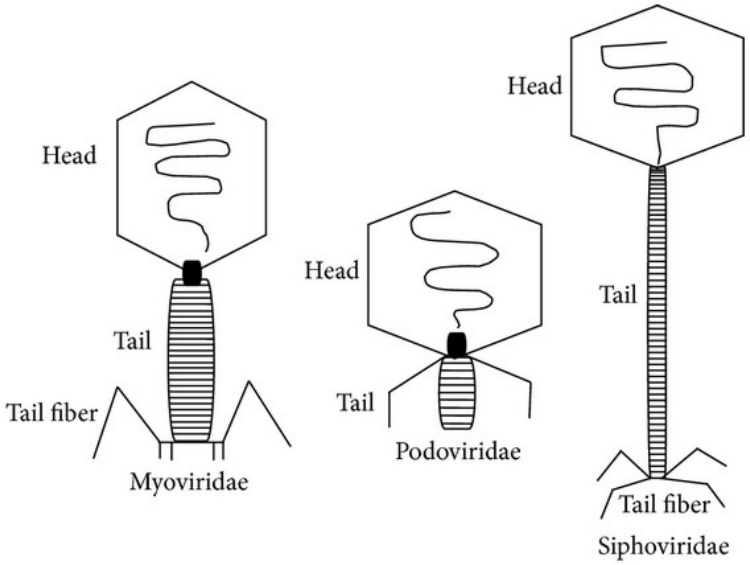
A common characteristic of phages is that, although their genome carries the information required to drive their own multiplication, they completely depend on the energy and protein biosynthetic machinery of their bacterial hosts to complete their life cycle, rendering them obligatory intracellular parasites of bacteria. The first contact between a phage and its host happens randomly and is usually made between the receptor molecules of the host (e.g., teichoic acid in Gram-positives or lipopolysaccharide in Gram-negatives) and specific phage proteins located at the tip of the tail fiber, or at one end of a filamentous phage. Injection of DNA follows immediately after a phage has stably and irreversibly adsorbed to the cell surface. Based on their subsequent propagation cycle, most phages can be broadly divided into two major groups: virulent and temperate. Virulent phages immediately redirect the host metabolism toward the production of new phage virions, which are released upon cell death within several minutes to hours after the initial phage attachment event (lytic cycle). Virulent phage infection results in clear plaques on the respective host bacterial lawns. Temperate phages can replicate either by the lytic cycle as

described above or by establishing a stable long-term viable relationship with their host bacteria, while the phage DNA is replicated together with the host's chromosome, during which viral genes that are detrimental to the host are not expressed (lysogenic cycle) [83].

As the vast majority of phages, the Staphylococci phages known so far are double-stranded DNA phages belonging to the *Caudovirales* order and to one of the three families: *Podoviridae* (class I), *Siphoviridae* (class II), and *Myoviridae* (class III). As suggested by the order name, all the phages belonging to these three families are composed of an icosahedral capsid and tail. The main differences between the members of the families are:

- *Podoviridae* have the shortest non-contractile tail, the smallest capsid and consequently the smallest genome (<20 kb);
- *Siphoviridae* have a long, flexible, non-contractile tail ended by a base-plate structure, a big capsid, and a genome of ≈ 40 kb;
- *Myoviridae* have a long contractile tail and the biggest capsid, containing a genome of >120 kb.

The other difference between the three families is crucial for selecting which phages can be used during the development of a phage therapy. Indeed, members of the *Podoviridae* and *Myoviridae* family are typically virulent phages, whilst *Siphoviridae* are characterized by having a lysogenic cycle [89]. Consequently, *Siphoviridae* are not the best choice for developing a phage therapy, though they can be useful to better understand CRISPR-Cas system functionality of Staphylococci strains.



The three-tailed phage families

Chapter 2

Comparative transcriptomic analysis of two clinical DAP-R/DAP-S *Staphylococcus aureus* isogenic couples by RNA-seq and bioinformatics

2.1 Aim of the study

This study reports the comparative transcriptome of DAP-R/hGISA (1-C) and DAP-R/qVISA (3-B) *S. aureus* clinical isolates *versus* their DAP-S/VSSA (1-A) and DAP-R/hGISA (3-A) isogenic counterparts.

The transcriptome, comprising of mRNAs and cis-encoding antisense small-RNAs, of these isolates was sequenced by the Illumina Mi-seq platform, and then analysed with the bioinformatics tool Rockhopper for a reference-based transcript assembly, followed by the quantification of transcript abundance and the test for differential gene expression.

The next step was a computational filtering analysis for sorting coding (mRNAs) and non-coding (sRNAs) RNAs differentially expressed in the DAP-R strains versus their DAP-S counterparts and, then, to identify only those presented in both DAP-R isolates to determine their RNA-signatures**.** Finally, bioinformatics and biological analysis were performed by using Gene Ontology, STRING and KEGG in order to clarify genes and targets function and involvement in bacterial antibiotic resistance to daptomycin and glycopeptides.

2.2 Materials and methods

2.2.1 Strains in study

Staphylococcus aureus strains used in this study consisted of two epidemiologically unrelated strain pairs of daptomycin-susceptible (DAP-S) and daptomycin-resistant (DAP-R) MRSA, isolated from distinct patients hospitalized in two different Italian hospitals. In details, the couple 1-A/1-C was isolated from skin infection at the "Ospedale Civico di Cristina Benfratelli" in Palermo; the couple 3-A/3-B was isolated from bloodstream infection at "Ospedali Riuniti di Bergamo". Each couple was isolated from a patient at different times, before and after the development of DAP-resistance emerging under DAP treatment. The antibiotic susceptibility was previously established determining the Minimum Inhibitory Concentration (MIC) by broth dilution, according to the Clinical and Laboratory Standard (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [90-92]. As shown in Table 1, isolates 1-A and 3-A were defined as MRSA/DAP-S, and isolates 1-C and 3-B as MRSA/DAP-R. Strains were also designed as Vancomycin-Susceptible *Staphylococcus aureus* VSSA (1-A), heterogeneous glycopeptide-intermediate *Staphylococcus aureus* hGISA (1-C and 3-A) and qVISA (3-B) by macro-Etest (MET). Each series, isolated from the same patient, with the initial pre-DAP therapy strain (1-A and 3-A) and its isogenic, isolated after the development of DAP-resistance during DAP treatment (1-C and 3-B), was previously assigned to the same pulsotype, MLST-type (MultiLocus Sequence Type) and SCC*mec*-type (Staphylococcal cassette chromosome *mec*) (Tab.1) [93].

2.2.2 RNA-sequencing

RNA-seq was performed using the Illumina Mi-seq sequencing system and two replicates were performed using two different libraries, a Single-end Library with 50bp reads (small-RNA Library) and a Paired-end Read Library with 150bp reads (mRNA Library), as a strategy to optimize the collected RNA-seq data according to the following protocols.

- Paired-end library RNA extraction

RNAs were extracted from the 4 samples grown until mid-log phase using the NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol with minor modifications. Bacterial cell pellets were lysed by the bead-beating procedure in the presence of 50 µl RA1 Buffer. Then 3.5 µl ß-mercaptoethanol was added and the lysate was filtered through NucleoSpin Filters (violet rings). RNA binding condition was adjusted by adding 350 µl of 70% ethanol to the lysate and the RNA was then extracted following the protocol. The quality of the total RNA was verified using a 2200 TapeStation RNA Screen Tape device (Agilent, Santa Clara, CA, USA) and its concentration ascertained using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Bacteria) from 2 µg of total RNA, and the depleted RNA was used as input in the Illumina TruseqRNA stranded kit without PolyA-enrichment. The prepared libraries were evaluated with the High sensitivity D1000 screen Tape (Agilent Tape Station 2200). The indexed libraries were quantified with the ABI9700 qPCR instrument using the KAPA Library Quantification Kit in triplicate according to the manufacturer's protocol (Kapa Biosystems, Woburn, MA, USA). Five µl of the pooled library at a final concentration of 2 nM was used for sequencing using Illumina Miseq with a 150 Paired-end Read sequencing module.

- Single-end library RNA extraction

Total RNA was extracted from the 4 strains grown until mid-log growth phase with Trizol reagent (Invitrogen) according to the manufacturer's protocol. After ribosomal depletion, sequencing libraries were prepared using the Illumina mRNA-seq sample preparation kit following the supplier's instructions except that total RNA was not fragmented, and double-stranded cDNA was size-selected (100 to 400 bp) to maximize also the recovery of small-RNAs.

The prepared libraries were evaluated with the High sensitivity D1000 screen Tape (Agilent Tape Station 2200). The indexed libraries were quantified in triplicates with the ABI7900 qPCR instrument using the KAPA Library Quantification Kit, according to the manufacturer's protocol (Kapa Biosystems, Woburn, MA, USA). From the pooled library, 5 µL at a final concentration of 4 nM was used for Miseq sequencing with an A single end stranded library with reads of 50bp sequencing module.

- Paired-end library preparation and sequencing

The samples were processed using the Illumina MiSeq technology, using an A paired end library with reads of 150bp and average insert size of 350/400 bp. After sequence data generation, raw reads were processed using FastQC v0.11.2 to assess data quality. The sequenced reads were then trimmed using Trimmomatic v.0.33.2 to remove only sequencing adapters for Paired-end reads.

A minimum base quality of 15 over a sliding window of 4 bases was required. Only sequences with length above 36 nucleotides were included into downstream analysis. Only trimmed reads were included in the downstream analysis.

- Single-end library preparation and sequencing

The samples were processed using the Illumina MiSeq technology with an A single end stranded library with reads of 50bp. After sequence data generation, raw reads were processed using FastQC v0.11.2 to assess data quality. Reads were then trimmed using Trimmomatic v.0.33.2 to remove sequencing adapters for Single-end reads, requiring a minimum base quality of 15 (Phred scale) and a minimum read length of 15 nucleotides. Only trimmed reads were included in downstream analysis.

- Paired-ends and Single-end libraries analysis

Paired-ends and Single-end RNA-seq reads were mapped on *S. aureus* NCTC 8325 (CP000253.1) reference genome using Rockhopper v.2.03 (24,25), a bioinformatic tool specifically designed for the bacterial gene structures and transcriptomes. Analyses were run on default parameter settings with verbose output to obtain expression data. Rockhopper reports the expression level for each transcript using RPMK (Reads Per Kilobase Million) value, except that data are not normalised by the total mapped reads but by upper quartile of gene expression. Rockhopper performs a statistical test for the null hypothesis, the hypothesis that states there is no differences between the expression of the transcript in the two conditions. The tool then computes a *p*-value for the differential expression of each gene (the probability of the observed data when the null hypothesis is true, i.e. the probability of observing a transcript's expression levels in different conditions by chance), and it reports the *q*-values, which are adjusted *p*-values, that control the false discovery rate using the Benjamini-Hochberg procedure. *q*-value indicates differential expression between experimental conditions, overcoming the problem deriving from multiple testing; Rockhopper considers *q*-values less than 0.01 (false discovery rate <1%)

to be significant. Finally, filtering analyses were computationally carried out for sorting, first, the differentially expressed RNAs in the DAP-R/hGISA and DAP-R/qVISA strains versus their parents and, then, to select only those present contemporarily in both DAP-R isolates showing the same up- or down-regulation trend.

2.2.3 Gene Ontology, KEGG, STRING, COG

In order to describe the biological processes, molecular functions, cellular components, pathways, protein class and ortholog genes for coding and non-coding RNAs resulting from RNA-seq analysis, GO term by Gene Ontology Consortium, KEGG, STRING and COGs classification were used. The Gene Ontology (GO) is a bioinformatic project, which provides a set of controlled vocabularies (ontology) to overcome the lack of a universal standard terminology in the field of biology, to provide detailed annotation of orthologous genes across a number of reference genomes, making communication and sharing data about genome functional elements easier [94]. It collects information across database, providing description of gene product and classifying them according to the cellular components, molecular functions, and biological processes in which they are involved. “Cellular component” refers to the cellular compartment, or the extracellular environment, where the gene product is active; “molecular function” is defined as the biochemical activity of a gene product; “biological process” refers to a set of molecular events to which the gene contributes [95,96]. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System is part of the Gene Ontology Reference Genome Project and was designed to classify proteins (and their genes) in family and subfamily, to facilitate high-throughput analysis. PANTHER can also predict the function of uncharacterized genes, exploiting the evolutionary relationships with known genes. KEGG (Kyoto Encyclopedia of Genes and Genomes) collects information from transcriptomics, epigenomics, metabolomics and proteomics to assign functional meanings to genes and genomes both at the molecular and higher levels. KEGG PATHWAY database was used to elucidate filtered RNAs role in bacterial metabolism [97]. Furthermore, RNAs were analysed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database, which is dedicated to functional associations between proteins, in order to integrate the description of their functions [98]. STRING assigns a confidence score (low

confidence: scores <0.4; medium: 0.4 to 0.7; high: >0.7) to each predicted association and integrates them in a final combined score. Together with the GO classification, RNAs were categorised according to the Clusters of Orthologous Groups (COGs), a classification based on comparison of protein sequences, useful for study of evolutionary relationships [99].

2.3 Results

The transcriptomes of two DAP-R/hGISA (1-C) and DAP-R/qVISA (3-B) unrelated Italian clinical isogenic isolates and their DAP-S/VSSA (1-A) or DAP-S/hGISA (3-A) counterparts were compared to determine their RNA-signatures and find traits that could be related with daptomycin resistance.

RNA-sequencing was performed using two different libraries to recover all the RNAs and do not lose the big or the small ones. The subsequently analysis of the transcriptomes derived from the two libraries required different steps. The first investigation was conducted with Rockhopper to annotate the transcriptomes on the reference genome and obtain the expression level of each RNAs. The second step was the selection of the statistically significant differentially expressed RNAs, showing a *q*-value ≤0.01, according to the tool's guidelines. Then, the RNAs differentially expressed in the DAP-R versus their DAP-S counterparts were computationally filtered to find only those present contemporarily in both DAP-R isolates.

The biological processes, molecular functions, cellular components, pathways, protein class and ortholog genes for selected RNAs were analyzed by using Gene Ontology Consortium (describing gene function, and relationships), PANTHER (Classification System), STRING (the predicted interactions protein-protein) and KEGG (pathways).

2.3.1 Paired-ends library summaries

From the Paired-ends library, the Rockhopper summary of the single DAP-R vs DAP-S pair showed that in 1-A strain, there were 853091 total reads and 826706 (97%) successfully aligned to the *Staphylococcus aureus* NCTC 8325 reference genome, as well as in 1-C strain there were 873327 total reads and 847504 (97%) successfully aligned to the same reference genome. Furthermore, in both strains the 50% of the reads were aligned in sense to miscellaneous RNAs whereas the 48% were aligned in antisense to miscellaneous RNAs, and the 1% of reads were aligned to unannotated regions. In 3-A strain, 101051 reads survived to the trimming process, of which 986353 (98%) successfully aligned to the reference genome, and in the 3-B strain there were 1011411 total reads and 979229 (97%) successfully aligned to the reference genome. Moreover, in both strains the 49% of the

reads aligned in sense to miscellaneous RNAs, on the contrary the 46% and the 47% aligned in antisense to miscellaneous RNAs respectively in 3-A and 3-B strain, and the 1% of the reads aligned to unannotated regions. Besides, the 1-A/1-C strains had thirteen 5'-UTRs and three 3'-UTRs, eight not antisense and nineteen antisense mRNAs, 341 differentially expressed protein coding genes, 1057 likely operons and 538 multi-gene operons; the 3-A/3-B strains had ninety-nine 5'-UTRs and seventy 3'-UTRs, eleven not antisense and twenty-two antisense mRNAs, 210 differentially expressed protein coding genes, 1122 likely operons and 547 multigene operons (Tab.2A).

2.3.2 Single-end library summaries

From the Single-end library, the Rockhopper summary of the single DAP-R *vs* DAP-S showed for 1-A strain a total of 304494 reads survived to the trimming with 59188 reads (19%) successfully aligned to the reference genome, and for the 1-C strain there were a total of 195956 reads, but only 35234 (18%) successfully aligned to the reference genome. Furthermore, in 1-A/1-C strains, the 80% and 86%, respectively, were aligned in sense to miscellaneous RNAs, whereas the 1% and 0% were aligned in antisense to miscellaneous RNAs, as well as the 5% and 7% were aligned to unannotated regions. With regard to the 3-A strain, a total of 429270 reads derived from the process, of which 156388 (36%) successfully aligned to the reference genome, whilst for the 3-B strain there were a total of 363244 reads and only 51519 (14%) were successfully aligned to the reference genome. In 3-A/3-B strains, the 53 % and 80% of the reads, respectively, were aligned in sense to miscellaneous RNAs, and the 1% and the 0% were aligned in antisense to miscellaneous RNAs, whereas the 7% and the 5% aligned to unannotated regions. Furthermore, 1-A/1-C strains had ten 5'-UTRs and five 3'-UTRs, 447 not antisense and 852 antisense RNAs, 220 differentially expressed protein coding genes, 1039 likely operons and 533 multigene operons. Finally, 3-A/3-B couple had thirty-eight 5'-UTRs and eleven 3'-UTRs, 814 not antisense and 1655 antisense RNAs, 416 differentially expressed protein coding genes, 1069 likely operons and 544 multigene operons (Tab.2B).

2.3.3 Coding RNAs

Fifty-three protein coding RNAs exhibited a statistically significant different expression in DAP-R isolates vs DAP-S ones, 9 were up-regulated and 44 down-regulated. They were obtained from both the libraries (Tab.3).

The biological processes in which these mRNAs were involved in concerned: cell wall biosynthesis, metabolism, nucleic acid and ribosomal metabolism, stress response, transport, and cell adhesion.

With regard to the cell envelope biosynthesis and organization (GO:0071554), 4 mRNAs involved in the peptidoglycan biosynthesis, 2 mRNAs included in cell-wall autolysis, and 1 involved in quality control of membrane proteins were differently expressed in both DAP-R strains compared to their parents.

Concerning the peptidoglycan biosynthesis, were down-regulated in DAP-R: *murF* (locus tag SAOUHSC_02317), encoding the UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase, *menH* (SAOUHSC_00984) encoding the 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase and predicted as functionally related to the same MurF, a gene for a hypothetical protein (SAOUHSC_00751) neighbouring and thus predicted functionally related to the UDP-N-acetylenolpyruvoylglucosamine reductase MurB ($STRING_{score}$:0.805), and *yycH* (SAOUHSC_00022) negative regulator of the two-component regulatory system (TCRS) WalKR.

For the cell-wall autolysis, according to the results, *cidB* (SAOUHSC_02850), which increases the activity of extracellular murein hydrolases, was up-regulated, conversely *lytS* (SAOUHSC_00230), member of the TCRS LytS/R, was down-regulated.

As regards to membrane proteins, the results showed a down-regulation in *ftsH* (SAOUHSC_00486), encoding ATP-dependent zinc metallopeptidase involved in the control system of integral membrane proteins quality. It was also found a different expression of other RNAs coding proteins that, according to Gene Ontology, are integral components of membrane (GO:0016021), with the following expression profile: over expression of one membrane spanning protein (SAOUHSC_03035), a down-regulation of the membrane proteins (SAOUHSC_01919, SAOUHSC_02376) and one down-regulated uncharacterized protein localised in the cell membrane (SAOUHSC_02391).

For the metabolic process (GO:0008152), many statistically significant differentially expressed mRNAs involved in different metabolic pathways were found. For the generation of precursor metabolites and energy, an up-regulated *ldh*2 encoding the L-lactate dehydrogenase (SAOUHSC_02922), involved in the lactic fermentation, and a down-regulation of the pyruvate kinase coding gene *pyk* (SAOUHSC_01806) involved in the glycolysis, were found. Furthermore, the DAP-R strains were characterized by a down-regulation of the protoporphyrinogen oxidase (SAOUHSC_01969), associated with the oxidation of respiratory electron transport chain enzyme, and of genes encoding for proteins involved in metabolism of several compounds. They were, in particular, a gene for the cysteine desulfurase (SAOUHSC_01727), involved in sulphur compound metabolic process, *hemC* (SAOUHSC_01774) for porphobilinogen deaminase, implicated in nitrogen compound (in particular porphyrin-containing compounds) metabolic processes, a sugar phosphatase (SAOUHSC_02396), an inositol monophosphatase (SAOUHSC_01055),a gene encoding an ABC transporter (SAOUHSC_00847) (the last three proteins are involved in the metabolic processes of phosphate-containing compounds), a lipoyl synthase (SAOUHSC_00861), and *moaA* (SAOUHSC_02536) encoding for molybdenum cofactor biosynthesis protein A, both involved in coenzyme and cofactor metabolism.

Regarding the nucleotide metabolic process, including purine and pyrimidine biosynthesis, the down-regulation of *deoB*, encoding phosphopentomutase (SAOUHSC_00101), of the GMP reductase *guaC* (SAOUSH_01330), of *carA* (SAOUHSC_01169), that encodes the carbamoyl-phosphate synthase small chain, and of the uridylate kinase *pyrH* (SAOUHSC_01235) was significative.

Concerning the amino acid catabolic process, the data indicated a down-regulation of the alanine dehydrogenase *ald*2 (SAOUHSC_01818), involved in amino acid catabolic processes, and of the lipase *lipA* (SAOUHSC_03006), involved in lipid catabolic processes.

For the cellular protein modification process, just the down-regulation in *nrd*I (SAOUHSC_00741), encoding the ribonucleotide reductase stimulatory protein, was found in both the DAP-R strains.

As regards to nucleic acid metabolic process (GO:0090304), data analysis showed down-regulated coding mRNAs for a DNA-binding protein (SAOUHSC_01693) involved in DNA repair, and for two proteins involved in the replication process that are the subunit δ

of the DNA polymerase III (SAOUHSC_01690), and the ATP-dependent DNA helicase PcrA (SAOUHSC_02123). With regard to RNA transcription and its regulation, the down-regulation of the DNA-binding protein HU (SAOUHSC_01490) and *nusB* (SAOUHSC_01621), encoding a transcription anti-termination protein, was characteristic of the DAP-R strains, as well as the down-regulation of the tRNA-dihydrouridine synthase (SAOUHSC_00031) and of *gatB* (SAOUHSC_02116) for the aspartyl/glutamyl-tRNA amidotransferase subunit B, both involved in tRNA metabolic process. For the ribosome biogenesis (GO:0042254) and maturation, an up-regulation of *engA*, encoding the ribosome-associated GTPase (SAOUHSC_01455), and a down-regulation of *rsgA* (SAOUHSC_01188), for putative ribosome biogenesis, were found.

For the SOS response machinery, a gene (SAOUHSC_01334) functionally related to LexA transcriptional repressor which inhibits genes involved in the response to DNA damage was up-regulated.

As concerns the stress response (GO:0006950), the DnaJ (SAOUHSC_01682), a chaperone protein preventing the aggregation of stress-denatured proteins in response to hyperosmotic condition and heat shock, was up-regulated. It was also observed an up-regulation of the luciferase-like monooxygenase (SAOUHSC_00304) involved in oxidative stress response, and of the poly-3-hydroxybutyrate depolymerase (SAOUHSC_00406), a protein that determines the PHB use as carbon source and energy storage in starvation condition.

A down-regulated globin domain protein with nitric oxide dioxygenase activity (SAOUHSC_00204) involved in nitrosative stress response existed in both the DAP-R.

Concerning the transport (GO:0006810), data showed a down-regulation of three genes encoding uncharacterized proteins with transporter activity (SAOUHSC_00099, SAOUHSC_00137, SAOUHSC_02700), of the Na^+/H^+ antiporter *mnhB*1 (SAOUHSC_00888), and of an ABC transporter with metal ion transport activity (SAOUHSC_00634) involved in cell adhesion. Only one up-regulated gene involved in transport, in detail for an inorganic phosphate transmembrane transporter (SAOUHSC_01387), was found. Furthermore, the filtering analysis highlighted the presence of three down-regulated genes involved in carbohydrate transport: putative maltose ABC permease (SAOUHSC_00177), putative mannitol-specific PTS system

component (SAOUHSC_02400) and N-acetylmuramic acid transporter belonging to the phosphotransferase system (PTS) (SAOUHSC_00158).

Finally, about the cell adhesion (GO:0007155), only the down-regulation of *sdrD* (SAOUHSC_00545), a gene that encodes the serine-aspartate repeat-containing protein D, was evidenced.

In addition, an up-regulated gene for a conserved hypothetical protein (SAOUHSC_00826) of unknown function was found.

2.3.4 Non-coding RNAs

RNA-seq data and subsequent filtering, highlighted 31 cis-encode antisense non-coding small-RNAs, 5 up-regulated and 26 down-regulated, all deriving from the Single-end library (Tab.4).

The predicted sRNAs were involved in different biological processes including the cell-wall biosynthesis and organization, metabolism, nucleic acid metabolism, stress response and transport.

As regards to cell-wall biosynthesis and organization, a sRNA involved in peptidoglycan biosynthesis was found. It was an up-regulated antisense sRNA for the gene *murA* (SAOUHSC_02337), that encodes the UDP-N-acetylglucosamine 1-carboxyvinyltransferase.

Data analysis showed several different small antisense non-coding RNAs implicated in diverse steps of the cell metabolism. In particular, an up-regulated antisense sRNA for the *odhA* gene (SAOUHSC_01418), encoding the 2-oxoglutarate dehydrogenase implicated in Tricarboxylic Acid Cycle and consequently in the aerobic metabolism processes, and 4 down-regulated sRNAs for genes involved in catabolic processes, i.e. the catabolism of the purine (SAOUHSC_01330), the superoxides (SAOUHSC_00093) and glutamine (SAOUHSC_00500) and for the proteolysis (SAOUHSC_00912). Still about the metabolism, several down-regulated sRNAs for genes involved in biosynthesis processes were found, i.e. biosynthesis of cholesterol (SAOUHSC_02860), cathecols (SAOUHSC_00906), folates (SAOUHSC_02543), vitamin B6 (SAOUHSC_00500), and pyridoxal 5'-phosphate (SAOUHSC_00500). Furthermore, an up-regulated sRNAs for formimidoyl-glutamase *hutG* ortholog (SAOUHSC_02610) involved in the histidine

catabolic process to glutamate and formamide, was a characteristic trait of both the DAP-R strains.

As concerns the nucleic acid metabolism, data highlighted the presence of some predicted small non-coding antisense RNAs complementary to genes implicated in different functions.

Particularly, thirteen down-regulated antisense sRNAs for: i) the *lexA* repressor (SAOUHSC_01333) responsible for the SOS response coding primarily for error-prone DNA polymerases, DNA repair enzymes and cell division inhibitors; ii) a ssDNA-binding protein (SAOUHSC_00349) implicated in the DNA metabolic process; iii) the Ribonuclease J1 (SAOUHSC_01035) responsible for the RNA metabolic process as the regulation of mRNA and rRNA decay; i) the 5, 16 and 23s ribosomal DNA and a tRNA-Val transcription (SAOUHSC_R0001, SAOUHSC_T00059, SAOUHSC_R00016, SAOUHSC_R0002, SAOUHSC_R0007, SAOUHSC_R0008, SAOUHSC_R0003, SAOUHSC_R0009, SAOUHSC_R00010, SAOUHSC_R0005).

For the oxidative stress response, only two down-regulated sRNAs were found, one for the *sodM* (SAOUHSC_00093), encoding the superoxide dismutase, and one for the *msrB* oxide-reductase (SAOUHSC_01431), involved in the antioxidant defence and to prevent oxidative damage.

For the heat, cold, nutrient limitation, and stress response, the analysis showed one down-regulated sRNAs for the ATP-binging subunit ClpB (SAOUHSC_00912), responsible for the re-solubilized protein aggregates during stress conditions, and one for the cold shock-like Protein CSPF (SAOUHSC_03045).

Finally, about the transport, a down-regulated antisense sRNA (SAOUHSC_00738) for a peptide transporter and an up-regulated antisense sRNA (SAOUHSC_1354) for a sodium ion transport alanine transporter were found.

2.4 Discussion

MRSA – together with *Enterococcus faecium, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp* acronymically termed "ESKAPE" pathogens – is a worldwide issue. These super bugs represent the most important public health challenge due both to continuous antibiotic resistance development and to the lack of new antibiotics. Understanding their resistance mechanisms, pathogenicity and virulence is key to successfully treat and control these "ESKAPEing" infections. The employment of powerful tools such as the NGS to study the transcriptome of important pathogens as *S. aureus* can lead to deep improvements in the knowledge on the biology of this MDR microorganism, although a consistent biological and bioinformatics background is required to elaborate the large amount of data (big data) resulting from RNA-seq analysis. Comparative microbial transcriptomics can help identifying differentially expressed genes related to various characteristics such as the susceptibility to specific antimicrobials. Furthermore, the RNAs can be used as markers and new molecular targets to the identification and control of infectious diseases. Particularly, the small bacterial RNAs have a known role for up- or downregulating gene expression and they play a critical role in all aspects of bacterial physiology. To date, there are only predictive in silico studies: although in bacteria were found hundreds of sRNAs, their role needs to be clarified.

The goal of this study was to define the transcriptome profiling of DAP-R *S. aureus*, performed by RNA-seq, investigating two isogenic pairs of DAP-R/S MRSA, to find out the differentially expressed genes presented in both DAP-R *S. aureus* in study. This study is meaningful for the research in this field since it compares DAP-R/S clinical isogenic MRSA, recovered from the same patient, in which the DAP-R strains emerged under daptomycin therapy, and it is not based on the study of DAP-R/S strain pairs generated by in vitro serial passages through increasing concentrations of daptomycin. Moreover, the two DAP-R MRSA pairs in study are not epidemiologically related and presented a very different genomic background in terms of MLST and PFGE profile, meaning that the transcriptomic similarity was closely related to their DAP-resistance therefore independent to strain-to-strain variations.

Here, are identified the statistically significant differentially expressed coding and non-coding RNAs in the DAP-R strains versus their DAP-S parents in order to focus the transcriptomic distinctive daptomycin-resistance traits of MRSA.

Comparative transcriptomic data highlighted differentially expressed RNAs involved in the biosynthesis and organisation of the cell-wall charactering the DAP-R isolates.
In particular, in DAP-R isolates some genes encoding enzymes involved in the different steps of peptidoglycan synthesis pathway, as the N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase MurF, the 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase MenH functionally related to MurF, the uncharacterized protein SAOUHSC_00751 functionally related to *murB*, the N-acetylmuramic acid transporter and the WalKR negative regulator YycH showed a down-regulation in the DAP-resistant strains. Therefore, data showed that the DAP-R strains presented a down-regulation of the genes involved in the biosynthesis of the peptidoglycan precursors and an up-regulation of the TCRS WalKR (originally YycGF), by the down-regulation of its WalKR negative regulator YycH, involved in the regulation of cell wall metabolism-associated genes and autolysis. For the peptidoglycan synthesis, it was also found a down-regulation of *menH*, functionally related to MurF and involved in Menaquinone and Phylloquinone epoxide biosynthesis. Menaquinone is a component of the staphylococcal membrane produced through the terpenoid-quinone pathway and required for a correct electron transport chain (ETC) functionality. Staphylococci characterized by an antibiotic-resistant phenotype have shown defects in ETC due to the inability to synthesize menaquinone [100], thus the down-regulation of the down-regulation of *menH* may be also related to the resistance acquisition at different levels. Besides, an up-regulated antisense small-RNA to the *murA* gene, involved in the pathway of peptidoglycan biosynthesis and, consequently, of the cell-wall because the MurA protein is responsible for the addition of enolpyruvyl to UDP-N-acetyl-glucosamine, was found. This result supports a possible role of the sRNAs in the regulation of the peptidoglycan biosynthesis and cell-wall organization that as it is knows represent a key role in the mechanism of resistance towards glycopeptides but, as suggested by our data, also in daptomycin resistance. The found sRNA should determine an accumulation of NAG and an alteration in the pathway of peptidoglycan biosynthesis.

The regulation of the autolysis represents a crucial point evidenced by obtained data, together with the induction of WalKR, it was found the concomitant *lytS* down-regulation and *cidB* up-regulation. LytS/R is the key of an autolysis regulatory pathway responding to cell membrane electrical potential [101] required for the expression of the extracellular murein hydrolase inhibitor, *lrgAB* [102]. *lrgAB* probably acts through its antiholin activity towards the holin-like proteins CidA and CidB [102] which conversely increase the activity of extracellular murein hydrolases possibly by mediating their export via hole formation. Therefore, the *lytS* down-regulation and *cidB* up-regulation in the DAP-resistant isolates indicated an enhanced activity of the extracellular murein hydrolase, particularly of CidB modifying the cell wall architecture.

The *ftsH* down-regulation can determine a low amount of the putative cell division protein FtsH, a metalloprotease anchored to the membrane. FtsH is thought to be involved in the quality control of cytoplasmic and integral membrane proteins, degrading the damaged ones, and acting as chaperone to help the folding [104]. About this function, is remarkable its STRING predicted association with two heat shock proteins: GrpE and HslO (both the associations have a combined score ≥ 0.7) (Fig.2). GrpE acts in response to hyperosmotic and heat shock stress by preventing the aggregation of denatured proteins; HslO is a redox regulated molecular chaperone, neighbouring to FtsH, which protects thermally unfolding and oxidatively damaged proteins from irreversible aggregation, defending toward oxidative stress. Staphylococcal FtsH shares 88% and 70% of identity with cell division protein FtsH of *Streptococcus pneumoniae* and *Bacillus spp.* respectively, and it is responsible for the PBP incorporation into cell membranes.

Concerning the genes responsible for the metabolism, data strongly indicate that the DAP-R *S. aureus* presented metabolic adaptations. First, the fermentative metabolism seems to be increased with respect to the aerobic one due to the up-regulation of lactate dehydrogenase gene, the already down-regulation of expression of ECT oxidase and the down regulation of pyruvate kinase, leading to an ATP deficiency because it catalyses a substrate-level phosphorylation. Further, the transition to a daptomycin non-susceptibility phenotype would alter amino acid, lipid, pyrimidine, and purine metabolism. Alterations in

the last two-mentioned metabolic pathway have been previously reported in *S. aureus* strains with reduced susceptibility to daptomycin [105].
Even several sRNAs targeting metabolism genes were found, i.e. an up-regulated antisense small-RNA to *odhA* gene involved in the TCA. Recent studies showed that a dysfunction of the TCA confers increased resistance to β-lactam antibiotics in *S. epidermidis* and an enhanced resistance to oxidative stress and a more positively charged cell surface [105]. We can speculate that the TCA dysfunction presented in these isolated might lead to increased daptomycin and glycopeptides resistance as a tolerance mechanism to these antibiotics activated probably by the diminution of the TCA cycle activity, possibly related also with a no susceptibility to the reactive oxygen species (ROS). Many different down-regulated sRNAs for catabolic and biosynthetic pathways are also typical traits of DAP-R isolates in this study.

With regard to the nucleic acid metabolism, some enzymes (polymerase) and building blocks (nucleotides) required for DNA replication seem to be produced in low amount, which, together with the down-regulation of cell division protein *ftsH*, suggest a slower growth of the DAP-resistant isolates compared to the susceptible ones. Our resistant isolates showed also a diminished expression of genes involved in transcription and translation. Data on ribosome metabolism strangely showed a decreased biogenesis of this complex molecular machine and an increase in its maturation control.

The up regulation of SOS response gene (SAOUHSC_01334, annotated from Mesak et al. as *sosA*) could be related to a block of the SOS response. *sosA* appears to be LexA regulated [107] exhibiting an up-regulation similar to that of the SOS genes [108]. LexA inhibits the SOS response until a DNA damage occurs; then RecA interacts with it generating an autocatalytic cleavage, which disrupts its DNA-binding part of LexA, leading to de-repression of the SOS regulon and eventually DNA repair. SOS-response block, together with the down-regulation of a gene involved in DNA repair, can cause strain hypermutation that is a key factor in development of mutational antimicrobial resistance.

Interestingly, we also detected some sRNAs for acid nucleic metabolic genes, i.e. to the above-mentioned SOS response system LexA repressor, to a ssDNA binding protein, to the Ribonuclease J1 and to 5, 16 and 23 S ribosomal DNA and tRNA-Val transcription.
These data also suggest that a SOS-response block leads to the hypermutator strains strongly related to the antibiotic resistant phenotype and an increased production of ribosomal components and control of mRNA and rRNA decay

For the oxidative stress response, the up-regulation of one monooxygenase SAOUHSC_00304 could be related with generation of reactive oxygen species (ROS), due to the occasionally oxygen incomplete reduction on interaction with these flavoproteins [109]. Moreover, the lower production of globin domain containing protein with nitric oxide dioxygenase activity (SAOUHSC_00204), which catalyses the conversion of nitric oxide (NO to nitrate NO^{-3}), could determine an accumulation of another reactive oxidant as NO. Taking together, these evidences suggest a potential role of reactive oxidant species production in development of daptomycin resistance. DAP-resistant strains showed also other stress response genes with altered expression compared to the susceptible ones. In particular, the down-regulation of *dnaJ* should lead to a diminished production of the chaperone protein DnaJ involved in the response to hyperosmotic and heat shock stress preventing or restoring aggregation of denatured proteins. DnaJ binds the unfolded or misfolded protein and acts as co-chaperone of DnaK, which hydrolyses ATP to ADP to correct fold the protein. Another co-chaperone is required for fully efficient folding, GrpE that acts as a nucleotide exchange factor, stimulating ADP release from DnaK and that has been already cited due its predicted STRING relation with the down-regulated *ftsH*. Collectively considered, these data suggest some alterations in the stress response of our resistant isolates compared to the susceptible ones, especially as far as it is concerned to the quality control of stress-denatured proteins. Lastly, we highlighted the overproduction of PHB depolymerase, indicating a utilization of different carbon sources in stress and nutrient limitation conditions, which could facilitate an improved survival of the DAP-R *vs* DAP-S strains in starvation and stress status.
In response to oxidative stress, we identified also two down-regulated antisense small-RNAs, one for *sodM*, encoding superoxide dismutase, and the other one for *msrB*, an

oxide-reductase involved in the antioxidant defense and in the prevention of oxidative damage. These data could suggest that the involvement of the oxidative stress response enzymes in the daptomycin resistance responsible for the survival of the cell due to an increased activity of ROS detoxification.
Other different sRNAs are involved in stress responses to heat and cold shock linked to important phenotypic properties including resistance to antimicrobials.

Regarding the transport, the up-regulation of the inorganic phosphate transmembrane transporter could be meaningful due to its STRING predicted relation with *walR*, *arlR* and *ssrA* (Predicted Interaction: gene neighbourhood; score ≥ 0.65.) (Fig.3), while all other genes involved in transport showed a down-regulation. WalR is member of the already cited two-component regulatory system WalKR that regulates genes involved in autolysis, biofilm formation, cell wall metabolism and positively controls the cell wall-hydrolytic activity. ArlR is a DNA-binding response regulator, member of the TCRS ArlSR involved in the regulation of adhesion, autolysis, multidrug resistance, and virulence. SrrA is a DNA-binding response regulator, member of the TCRS SrrAB, which is involved in the global regulation of Staphylococcal virulence factors in response to environmental oxygen levels. SrrA binds to the *agr*, *spa* and *tst* promoters and represses their transcription.

Finally, as regards to cell adhesion our filtering analysis has selected only one gene, with a statistical differential expression between daptomycin resistant and susceptible isolates, encoding SdrD. The Serine-aspartate repeat-containing protein D is a *S. aureus* cell surface-associated calcium-binding protein with a possible interactions role with components of the extracellular matrix of higher eukaryotes. The altered expression of proteins associated with the cell surface could be related to, or directly a consequence of, the supposed modified structure of the cell membrane and cell wall of DAP-R *S. aureus*.

Chapter 3

Characterization of CRISPR-Cas system in Italian clinical MDR *Staphylococcus epidermidis*

3.1 Aim of the study

This study first reports the characterization and functionality of the CRISPR-Cas system in Italian pathogenic clinical MDR *S. epidermidis* strains, and their sensitivity to phage infection.

The entire CRISPR-Cas locus of selected strains was sequenced, and anti-plasmid activity of the CRISPR-Cas was tested by conjugation, in order to study the allowance of these strain to the plasmids acquisition.

Phage infection assays were performed with bacteriophages belonging to each of the three Staphylococcal phage families, to evaluate if a phage therapy can be effective and helpful in overcoming the antibiotic resistance issue.

3.2 Materials and methods

3.2.1 Strain collection and growth conditions

Clinical pathogenic *Staphylococcus epidermidis* strains in this study were selected for their resistance profile among the bacterial culture collections of the Medical Molecular Microbiology and Antibiotic Resistance Laboratory (MMARL) at the University of Catania. All isolates were identified and characterized for their resistances [110]. Selected strains were representative of different Pulse Field Gel profiles and Multi Locus Sequence Types.

Phages used to test the sensitivity came from the Dr. Hatoum lab. collection, and they were representatives of the three Staphylococcal phage families: *Podoviridae* phage Andhra [111], *Siphoviridae* phage CNPx [112], and *Myoviridae* phage ISP [113].

Bacteria, named IS (Italian Strains) 1 to 10 (Tab.5), were cultured at 37 °C.

Tryptic soy broth (TSB), tryptic soy agar (TSA), Brain Heart Infusion (BHI), and BHI-agar were used to culture bacteria and phages. Heart infusion agar (HIA) was used to prepare molten top agar halving the suggested concentration of powder. Media used in this study were purchased from Becton Dickinson (Franklin Lakes, New Jersey, United States).

3.2.2 Molecular characterization

PCRs performed to detect *cas*1 and *cas*2 genes and the *cfr*-carrying plasmid have been done on DNA extracted at the MMARL according to the method described by Pithcer [114] with minor modifications. Briefly, a bacterial colony was inoculated in 7 ml of TSB overnight. The culture was centrifuged at 3000 rpm for 15 minutes at room temperature and the harvested cells were resuspended in 100 µl of 10:1 TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and transferred into a fresh 2 ml microtube. To lysate the cells, the tube was incubated at 37 °C for one hour with lysozyme (10 mg/ml) and lysostaphin (100 µg/ml) at the final concentration of 2.5 mg/ml and 25 µg/ml respectively. A second lysis step was performed adding 550 µl of GES (5M Guanidinium thiocyanate, 0.1M EDTA pH 8.0 and 0.5% SDS) to the previous lysate and incubating the mixture for ten minutes at room temperature. A first precipitation step had been done adding 250 µl of cold 7.5M Ammonium acetate and letting the tube in ice for ten minutes. A RNase treatment was

performed by adding the enzyme at the final concentration of 100 µg/ml and incubating the lysate at 37 °C for 30 minutes. DNA was extracted with a volume of phenol–chloroform–isoamyl alcohol (25:24:1) and centrifuging the tube at 12000 rpm for 15 minutes. The DNA containing upper phase was transferred into a fresh 1.5 ml microtube and precipitated with 0.54 volumes of ice-cold isopropyl alcohol. After one-hour precipitation at -20 °C, the tube was centrifuged as above, the supernatant was carefully discarded, and the DNA was resuspended in dH_2O. An electrophoresis on 1% agarose gel was performed to visualize the extracted DNA. 1 µl of DNA was used as template for the following PCR reaction:

Final Volume: 25 µl

5X Colorless GoTaq® Reaction Buffer:	5.0 µl
(200 µM) dNTP:	2.5 µl
(10 pmol/µl) primer forward:	1.0 µl
(10 pmol/µl) primer reverse:	1.0 µl
5 u/µl GoTaq® DNA Polymerase:	0.12 µl
dH_2O:	14.38 µl

Amplification program:

- 1 cycle: 94 °C 1 minute
- 30 cycles: 53 °C 1 minute
  68 °C 1 minute (*cas*1/*cas*2 primers) - 3 minutes (*cfr* primers)
  94 °C 1 minute
- 1 cycle: 53 °C 30 seconds
  72 °C 10 minutes
  4 °C ∞

Other PCRs had been done to detect and sequence all the genes of the CRISPR locus, and they were performed in the Dr. Hatoum lab. at the University of Alabama according to the colony PCR protocol. Briefly, a well isolated Staphylococcal colony was resuspended in 200 µl of dH_2O and centrifuged at 13000 rpm for 1 minute. Than 1 µl of the cell pellet was resuspended into 40 µl of colony PCR buffer (see recipe below) plus 1 µl of lysostaphin (1

mg/ml). The resuspended colony was lysed by the following PCR program in a thermocycler:

- 37 °C, 20 minutes
- 98 °C, 10 minutes

Cells were harvested by centrifuging as above and 1 µl of the supernatant was used as template in the following PCR reaction:

Final Volume: 25 µl

5X Phusion Buffer:	5.0 µl
(10 mM) dNTP:	2.0 µl
(10 pmol/µl) primer forward:	1.0 µl
(10 pmol/µl) primer reverse:	1.0 µl
2 u/µl Phusion® High-Fidelity DNA Polymerase:	0.13 µl
dH_2O:	15.87 µl

Thermal profile:

- 1 cycle:	95 °C	1 minute
- 30 cycles	95 °C	10 seconds
	55 °C	10 seconds
	72 °C	1 minute per 1 kb length of PCR product
- 1 cycle	72 °C	twice the previous elongation time
	12 °C	∞

Colony PCR buffer:

- 250 mM KCl
- 50 mM Tris-HCl pH 9
- 5 mM $MgCl_2$
- 0.5% Triton X-100
- dH_2O to achieve the final volume

Used primers are listed in Tab.6.

3.2.3 Primer walking

To sequence the entire CRISPR locus, the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (PROMEGA, Madison, Wisconsin, United States) according to the manufacture's protocol and quantified using the NanoDrop 2000 (Termo Fisher Scientific, Waltham, Massachusetts, United State). 8 µl of the purified PCR product (12-25 ng/µl) was combined with 4 µl of the proper primer (10 pmol/µl) and sent to the Eurofins Genomics. Sequences were aligned to the pCRISPR-Cas reference sequence (derived from the *Staphylococcus epidermidis* RP62a) using the SnapGene software (v 3.1.4).

3.2.4 Phage infection assay

The capability of some phages to plaque on the Italian *S. epidermidis* strains was tested using the "molten top agar (HIA) layer" infection assay. For this purpose, liquid (60° C) top agar and bacterial overnight culture were combined, in a sterile glass tube, in a 40:1 ratio and the mixture was rapidly poured onto a pre-warmed 5 mM $CaCl_2$ TSA plate to create a top agar overlay. It was let undisturbed for 10 minutes on the benchtop to allow the top agar to set. Meanwhile, ten-fold serial dilutions (up to $1x10^{-7}$) of the phage were prepared starting from a high titer lysate (10^9-10^{10} PFU/ml). All the tests had a positive-control and a negative-control of infection.

All the phage assays were performed adjusting the media with $CaCl_2$ to 5 mM to ensure the best replication conditions for the phage.

3.2.5 Making competent cells

Bacterial cells were made electrocompetent starting from an overnight culture diluted 1:100 in fresh TSB media and incubated at 37 °C with shaking until reach the OD_{600} of 0.5 (exponential growth phase). Cells were pelleted by centrifuging at 5000 rpm for 10 minutes at 4 °C. All subsequent steps were performed in ice. The supernatant was completely discarded to remove all the growth media that can interfere with the electroporation generating electric arcs, and cells were resuspended in an equal volume of ice-cold water,

then centrifuged as above to wash them. The pellet was resuspended in a half-volume of ice-cold 10% glycerol water and centrifuged as above. Cells were finally resuspended in a volume of ice-cold 10% glycerol water equal to 0.015 times the original volume and saved at -80 °C as 50 µl stocks to be used in further experiments.

3.2.6 Bacterial transformation and transduction

To transform plasmids into *S. epidermidis*, an electrocompetent bacterial stock for each plasmid and one as negative control was gently thawed in ice, meanwhile 40 µl of the plasmid was dialyzed on 0.025 µm membrane filter (Millipore, Burlington, Massachusetts, United States) for 20 minutes. At the end of the dialysis, the dialyzed plasmid (sterile water for the negative control) was combined with the electrocompetent cells and the mixture was transferred into an electroporation cuvette and let it rests for exactly 10 minutes on the benchtop. Then, it was subjected to an electric shock using the Gene Pulser Xcell™ Electroporation Systems (Bio-Rad, Hercules, California, United States) with the following program: 21 KV/cm; 100 Ω; 25 uF.

After the electroporation, 500 µl of fresh, sterile TSB was quickly added to the mixture and the entire content of the cuvette was transferred into a 1.5 ml microtube and incubated at 37 °C with shaking. After 2 hours of incubation, 100 µl of bacterial suspension was streaked onto a 10 mg/l chloramphenicol TSA plate to isolate the transformants. Plates were incubated at 37 °C overnight. Colonies were checked out for the plasmid by PCR.

When transformation failed, plasmid transduction was performed co-culturing the CNPx phage with a phage-susceptible bacterial strain containing the desired plasmid at the MOI of 1. After culture clearing, phage lysate was recovered centrifuging the co-culture at 5000 rpm for 10 minutes and filtering the supernatant through 0.22 µm filters. Collected filtered phage lysate was co-cultured with the target *S. epidermidis* strain at 37 °C overnight and then cells were streaked onto 10 mg/l chloramphenicol TSA plates. Colonies were checked out for the plasmid by PCR.

Plasmids used in this study were:

- pAH011, carrying a CRISPR spacer that targets the nickase gene found on conjugative staphylococcal plasmid pG0400 (that also confers mupirocin resistance), and a chloramphenicol resistance gene (*camR*)

- pNB006, harboring *camR* and a CRISPR spacer that targets part of the Andhra phage genome.

3.2.7 MIC determination

Chloramphenicol and mupirocin MICs were obtained using the microdilution method for MIC determination according to the CLSI and EUCAST guidelines [90-92]. All the MIC assays had quality control strains. Antibiotic (i.e. chloramphenicol or mupirocin) was prepared starting from a 10000 mg/l stock solution, diluting it in sterile broth up to reach a concentration once higher than the highest limit of the range to test. Eleven 1:2 serial dilutions of the antibiotic were performed in the wells of a 96-wells microplate with conical bottom containing sterile broth. The last well of every row did not contain antibiotic and represented the growth positive control. Two to five well-isolated colonies from a fresh overnight culture were resuspended in sterile dH_2O or saline solution until reaching turbidity equal to the 0.5 McFarland standard or an OD_{450} = 0.6 (≈ $5X10^8$ CFU/ml). The bacterial suspension was first diluted 1:100 in sterile broth, and then diluted 1:10 in all the wells. One row of wells did not contain bacteria and it was the sterility control. The plate was incubated for 18 hours at 37 °C and the MIC value was confirmed as the lowest concentrations capable of inhibiting bacterial growth.

3.2.8 Conjugation assay

Anti-plasmid immunity was tested as published by Walker F.C. and Hatoum-Aslan A. [115] with minor modifications. On the first day, the "recipient" *S. epidermidis* strain to test and a CRISPR-ineffective control were streaked onto BHI agar plates containing 10 mg/l chloramphenicol to obtain single colonies. Plates were incubated at 37 °C overnight. The *S. aureus* RN4220 carrying the plasmid pGO400 (here onward called "donor") was inoculated into 2 ml of TSB containing 5 mg/l mupirocin starting from a freezer stock. The culture was incubated with shaking at 37 °C overnight.

On the second day, 3 large (> 1 mm in diameter), well isolated recipient colonies and 3 control colonies were inoculated into 15 ml centrifuge tubes containing 2 ml of 10 mg/ml chloramphenicol BHI. At the same time, the donor culture was diluted 1:100 into a 15 ml centrifuge tube containing sterile TSB with 5 mg/ml mupirocin. Recipients and controls

grew at 37 °C with shaking until they reach an OD_{600} greater than 2.0 (from 6 to 8 hours, depending on the strain). The amount of donor and recipient to combine to achieve a 1:4 ratio (recipients:donors) for filter mating was calculated dividing 200 by the measured OD_{600}, because at an OD_{600} of 1 there was approximately 8 x 10^8 CFU/ml of *S. aureus* and 2 x 10^8 CFU/ml of *S. epidermidis*. The calculation allowed to standardize the numbers of donors and recipients that had to been combined. The calculated volumes of recipients and donors were combined together into 1.7 ml microcentrifuge tubes and centrifuged at 6200 x g for 2 minutes to pellet the cells. The supernatant was discarded, and the pellets were resuspended in 1 ml of sterile BHI, then centrifuged as above to remove the antibiotics. Each donor-recipient pair pellet was resuspended in 100 µl of BHI broth and transferred onto the center of a 0.45 µm membrane filter already placed onto a pre-warmed BHI agar plate containing no antibiotics (up to 3 filters on a single plate). After the cell suspension fully dried on the benchtop, the plates were incubated upside-down at 30 °C overnight.

The filters were placed into 50 ml centrifuge tubes (one for each filter) containing 3 ml of BHI broth without antibiotics on the third day, and the tubes were vortexed to completely resuspend the cells. The cell suspensions were ten-fold serially diluted in sterile BHI broth with no antibiotics, out to the 10^{-7} dilution. 10 µl of each dilution was spotted onto a pre-warmed BHI agar plate containing chloramphenicol and another containing both chloramphenicol and mupirocin. Once the spots dried, the plates were incubated at 37 °C overnight.

The conjugation efficiency was evaluated at day 4. The number of colonies in the highest dilution where the colonies were still countable, both in the chloramphenicol and the chloramphenicol/mupirocin BHI agar plates, was counted and multiplied by 100 and by the dilution factor to obtain the CFU/ml value. The colonies grew in the presence of chloramphenicol only represented the “number of recipients”, whereas the colonies grew in the presence of both chloramphenicol and mupirocin represented the “number of transconjugants”. The *S. aureus* RN4220-pGO400 donor was not able to grow in the presence of chloramphenicol. Conjugation efficiency was calculated using the following formula:

$$Efficiency = \frac{number\ of\ transconjugants\ (\frac{CFU}{ml})}{number\ of\ recipients\ (\frac{CFU}{ml})}$$

A high efficiency corresponded to an inactive CRISPR system, while a lower efficiency indicated active CRISPR defense.

3.3 Results

3.3.1 CRISPR-Cas locus architecture and sequence

In the preliminary phase of this study, *cas*1 and *cas*2 genes were found in 4 out of the 10 strains, namely IS6, IS7, IS8, and IS10. The other two genes of the CRISPR-cas locus that were searched in all the Italian pathogenic *S. epidermidis* strains were *cas*6 [78] and *cas*10 (also known as *csm*1); the first was present in all of them, whereas the latter was detected in IS3, IS4, IS6, IS7, and IS8. The Repeat/Spacer (R/S) sequences localized before *cas*1 and after *cas*6 were found in 3 strains. Only the strains that were positive for both *cas*6 and *cas*10 and for the R/S sequences - i.e. IS6, IS7 and IS8 - were subjected to further analysis. All these strains had the *csm*2, *csm*3, *csm*4, *csm*5 and *csm*6 genes and the structure of the CRISPR-Cas locus was confirmed to be the typical of the *S. epidermidis* type III-A by amplifying and sequencing all the genes with primers that anneal in different regions of adjacent genes (data shown in Tab.7).

The comparative analysis of the CRISPR-Cas locus sequence of IS6 and IS8 with the sequence of *S. epidermidis* RP62a revealed only one SNP in the *csm*5 sequence, a synonymous substitution G192A (R64). The same analysis with IS7 highlighted 131 SNPs which are distributed all over the genes of the CRISPR-Cas locus: 75 SNPs were synonymous substitutions, whereas 56 were non-synonymous. All the SNPs and the related amino acid substitutions are listed in Tab.8.

With regard to the repeat/spacer sequences (R/S), both IS6 and IS8 had 9 spacers before *cas*10 (6 more than *S. epidermidis* RP62a) and 2 spacers after *cas*6, as well as *S. epidermidis* RP62a. All the spacer sequences were analyzed using the NCBI Basic Local Alignment Search Tool (BLAST), two of them had the same sequence of part of the IME1348_01 phage and CNPx phage genomes, whereas the remaining had unknown targets. The IS7 had 11 spacers before *cas*10 and 2 spacers after *cas*6, all of them with unknown targets.

3.3.2 Phage sensitivity

Phage infection assays revealed that only the IS7 was susceptible to the infection with the *Podoviridae* phage Andhra (Fig.1), and the IS1 and IS9 showed infection plaques when co-

cultured with the *Myoviridae* phage ISP, whereas all other combinations showed no phage plaquing on the tested strains (Tab.9).

3.3.3 MIC results

The calculated chloramphenicol MICs of the IS6, IS7 were 64 mg/l and 8 mg/l respectively, making impossible to isolate transformant cells, and it was 4 mg/l for the IS8, low enough to allow transformants isolation. Furthermore, mupirocin MIC of the IS7 was 256 mg/l, too high to consent conjugation assay with the pGO400 plasmid. Mupirocin MIC value of the IS8, instead, was less than 0.06 mg/l, making possible to test the anti-plasmid CRISPR activity by conjugation assay.

3.3.4 Anti-plasmid activity of IS8 CRISPR-system

Despite the impossibility of testing CRISPR anti-phage activity of the IS8 due to the lack of phages able to plaque on the strain, it was feasible to study its anti-plasmid activity. pAH011 plasmid was used due to the presence of the anti-nickase spacer and pNB006 plasmid was selected as a negative control. Plasmid transformation with the same plasmids already worked with the electrocompetent IS7 but failed several times with the IS8. Hence, plasmid transductions using the CNPx were performed, and the IS8-pAH011 and IS8-pNB006 colonies were isolated on TSA plates containing 10 mg/l chloramphenicol. Plasmid presence was confirmed by PCR.

As shown in figure 2, conjugation efficiency was similar and high for both the tested IS8-pAH011 and the negative control IS8-pNB006, indicating that the CRISPR system of the IS8 probably lacks the anti-plasmid activity, although its integrity.

3.4 Discussion

CRISPR system protects bacteria from attack by phages or plasmids [116] playing an important role in the maintenance of the equilibrium between phage-host arm race and bacterial evolution [117], because phages usually kill their hosts, but foreign DNA elements are fundamental for the acquisition of new advantageous characteristics as antibiotic resistances or virulent factors.

CRISPR locus is present in almost all archaeal genomes and about 40% eubacterial genomes according to the sequence data [118]. It consists of short repeated sequences (24–48 bp) separated by unique spacers with size ranged from 21 to 72 bp [119,120]. The CRISPR-Cas system also comprises the *cas* genes, a "leader sequence" adjacent to the CRISPR locus, and the spacers, short DNA sequences obtained from foreign DNA, such as phages and plasmids, that are inserted between repeated sequences. The composition and arrangement of spacers in bacteria likely reflects the interactions between bacteria and foreign nucleic acids in the environment [121].

Despite some papers already reported worldwide the CRISPR locus characterization of different bacterial species [122-125], we have no data about the presence, the structure, and the functionality of the CRISPR system in Italian pathological Staphylococci. As reported by Qiuchun Li *et al*. [126], nowadays CRISPR system has only been detected in a few Staphylococcal strains and there is still conflicting evidence as to whether the presence of CRISPR-Cas locus is an ancestral or acquired characteristic. Although sequence similarities between different Staphylococcal species suggest that it is ancestral, its patchy distribution and close association with SCC*mec* in the Staphylococci suggest that it is either mobile or easily able to be mobilized by other elements [127]. In all the Italian pathogenic *S. epidermidis* strains analyzed in this study, at least the *cas*6 gene was detected, suggesting two possible hypotheses: i) an ancient acquisition of the entire CRISPR locus and the subsequently loss of some genes or ii) a novel acquisition of single genes of the CRISPR system. Both hypotheses have strengths and weaknesses. As previous published [128], seems to be possible for CRISPR loci to be broken up and dispersed in chromosomes by transposons with the potential for creating genetic novelty. This could explain the presence of the *cas*6 gene in all the strains and why the IS10 has only the *cas*1 and *cas*2 genes, but it

is in contrast with the same identical structure of the type III-A CRISPR system found in all the *S. epidermidis* strains. The evidence that IS3 and IS4 were positive for the *csm*1, *csm*2, *csm*3, *csm*4, *csm*5, *csm*6 and *cas*6 but they lacked the *cas*1 and *cas*2 genes and the related R/S sequences could suggest the acquisition of the whole CRISPR locus and the secondary loss of some parts. They could have a so called "isolated *cas* locus" [129], a *cas* locus that does not have a nearby CRISPR array.

As revealed by sequencing, only the IS6, IS7, and IS8 strains had the whole CRISPR-Cas locus. Particularly, sequence analysis highlighted the same CRISPR locus aminoacidic sequence of the *S. epidermidis* RP62a in the IS6 and IS8, with only one synonymous SNP, and 132 SNPs in the IS7. Despite the high number of nucleotide substitutions, the IS7 has 48 alterations of the amino acid sequence scattered in all the genes of the CRISPR locus with the exception of *cas*2. Notably, an aminoacidic substitution affects the first methionine of the *csm*4 gene, it would be interesting do further studies on the functionality of the CRISPR system of this strain, but its multi-drug resistant profile makes really difficult to work on it.

The presence of more and new spacers in all the sequenced strains testifies a previous activity of Cas1 and Cas2, but, to date, nobody has been able to replicate in laboratory the exact conditions that allow the adaptation. The existence of the spacer targeting CNPx phage in the IS8, the inability of the same phage to plaques on that strain, and the successful transduction of the pAH011 and pNB006 plasmids inside the IS8 obtained using CNPx, show, at the same time, the presence of the phage receptors on the bacterium surface and a possible anti-phage CRISPR activity, although it is impossible to be sure of the absence of other defense mechanisms.

Interestingly, the strains in this study showed resistance to phage infection when co-cultured with a representative of each of the three Staphylococci phages families, indeed only the *Podoviridae* phage Andhra was able to plaque on the IS7, and the *Myoviridae* phage ISP plaqued on the IS1 and IS9. This suggests the evolutionary success of bacteria able to survive to the phage infection also in the human body, in which phage have been found in different clinical samples such as ascitic fluid and urine, in the peritoneal cavity and in the intestine, and even in serum [130,131], supporting the hypothesis that the CRISPR-Cas systems can shape the evolution of human microbiomes

influencing the allowance of bacterial cells to foreign DNA or their immunity to phage infection [132]. This could represent a challenge in the development of a phage therapy for the treatment of multidrug resistant pathogenic Staphylococci, although – luckily - the co-evolution of phages and their hosts is like a never-ending war, from which scientists can learn how to use even single phage components to overcome the serious public health problem represented by the antibiotic resistance.

As already mentioned, the multi-drug resistant nature of the pathogenic strains was an obstacle in the study of their CRISPR-Cas system activities. The IS7 was the only strain having the entire CRISPR-Cas locus and being susceptible to a phage, but its resistance to chloramphenicol made impossible to isolate transformants with the pNB006 plasmid harboring a spacer for Andhra phage, hence was not possible to study its anti-phage activity.

The IS8 had the whole CRISPR-Cas locus and it was sensible to chloramphenicol and mupirocin, but we did not find a phage able to plaques on it. However, it was possible to test its CRISPR anti-plasmid activity. For this purpose, two different transformants of the IS8 were made, one with the pAH011 plasmid carrying the anti-nickase spacer and the *camR* gene conferring resistance to chloramphenicol, and the other one with the pNB006 plasmid, carrying the same *camR* gene and the targeting Andhra spacer useful as negative control. The plasmids were introduced into the IS8 cells by transduction with the CNPx phage and positive colonies were isolated on TSA plates containing 10 mg/l of chloramphenicol and screened for the plasmids by PCR. Tested conjugation efficiencies of the CRISPR-Cas system of the IS8-pAH011 and IS8-pNB006 transformants were similar and high, indicating a lack of anti-plasmid function of the IS8 CRISPR-Cas system, although it has the whole locus and the genes have the identical sequences of the *S. epidermidis* RP62a, which has a well functional CRISPR-Cas system.

A non-functional CRISPR could still be useful because CRISPR genes seem to play a role also in the DNA repair, as already published [80]. Hopefully, when a phage able to infect the IS8 will be found, further studies about the anti-phage activity of its CRISPR-Cas system can be conducted by making a new plasmid harboring a spacer specific for the phage.

Summarizing, CRISPR characterization of 10 Italian pathogenic *S. epidermidis* strains, epidemiologically not related, highlighted a surprising prevalence of at least one gene of the CRISPR-Cas locus in all the strains, 2 isolated *cas* loci, and 3 whole type III-A CRISPR-Cas systems, showing a spread of CRISPR elements in all the strains. New spacers acquisition attested multiple adaptation events, useful to protect of bacteria against phages living in the human body. This could be one of the reasons behind the phage resistance found as a common trait in these strains, with only 3 of them susceptible to the phage infection with a representative of the three Staphylococci phages families. This data should be considered in the perspective of developing a phage therapy as a strategy to overcome the threat of the antibiotic resistance.

Nevertheless, it is possible to speculate that antibiotic therapy promoted the facile acquisition of foreign elements conferring antibiotic resistance, among other things, decreasing genome stability/increasing plasticity, and enabling the colonization of new habitats, including the antibiotic-laden hospital environment. This could be the reason behind the presence of broken CRISPR-Cas system in pathogenic, multi-drug resistant strains.

It is important to remember that CRISPR systems also avoid the acquisition of plasmid DNA that often harbors gene conferring antibiotic resistance, hence it seems to have both positive and negative correlations with some antibiotic resistances.

Finally, the multi-drug resistance profile of the pathogenic strains made possible to analyze only the anti-plasmid activity of the CRISPR system of one strain. Although the perfect match with the well-known *S. epidermidis* RP62a CRISPR-Cas locus and its completeness, it seems to be non-functional.

4. Conclusion

Both the studies reported in this doctorate thesis aim to deepen knowledge of the main traits characterizing Italian clinical multi-drug resistant Staphylococci, among which *S. aureus* was our first choice due to its clinical relevance and the worldwide spread of resistant strains. It is well known that *S. aureus* can acquire new antibiotic resistance determinants from other coagulase negative Staphylococci, in particular *S. epidermidis*. Despite it is a commensal that can be even helpful for our health, it remains the main source of genes responsible for virulence and antibiotic resistance among the Staphylococci. Due to their close relationship, *S. epidermidis* can easily transfer genes to *S. aureus*, but – luckily - the horizontal gene transfer from CoNS to *S. aureus* appears unidirectional. The lack of CRISPR sequences in these bacteria is responsible for such genetic exchanges, as recently demonstrated [133]. But not only the presence of genes is responsible for acquiring antibiotic resistance, indeed another paper addressed the role of gene expression in the development of antibiotic resistance and found, by using real-time qPCR, that *dlt*A over-expression is related to daptomycin resistance in *S. aureus* [93]. Nevertheless, these studies had some limits: the first investigated only one *S. epidermidis* strain, and in the second the real-time qPCR do not allow the analysis of the entire transcriptome, feasible, instead, by using the new RNA-seq technology. Indeed, although the big number of genomic studies, we still know little about the transcriptomes features, comprising mRNAs and small-RNAs, and about the CRISPR system in pathogenic Staphylococci, also because first RNA-seq study can be dated to 2008 [134,135] and the description of CRISPR as an adaptive immune system dates back to 2003 [136].

The implication of different RNA signatures involved in developing of resistance was, in our experimental plan, evaluated in a couple of DAP-R *S. aureus* by RNA-seq and our results, showed the contribution of Staphylococcal mRNAs and sRNAs in daptomycin resistance, with multiple pathways associated, including the cell-wall biosynthesis and organization, metabolism, nucleic acid metabolism, stress response and transport, confirming the role of transcriptome in developing antibiotic resistance.

With regards to the CRISPR system, it was rarely reported in Staphylococci [69], and the prototypical type III-A CRISPR–Cas system was first found in *S. epidermidis* RP62a. For

these reasons, we decided to use MDR *S. epidermidis* strains as model to learn more about Staphylococcal CRISPR system, and we found the type III-A CRISPR system in the 33% of the Italian pathogenic MDR *S. epidermidis*, confirming the identical CRISPR structure in all the *S. epidermidis* regardless of their geographical place of isolation. Moreover, our findings showed that MDR strains can also be CRISPR positive. It was recently discovered that the *S. epidermidis* type III-A CRISPR system targets both DNA and ssRNA, and also transcriptionally active regions of DNA [137]. Transcriptomic analysis together with our new knowledge about CRISPR system suggest us the possibility to use CRISPR, in the future, to target not only the genes, but also RNAs (messenger and small) to cure the antibiotic resistance. Furthermore, RNA-seq and CRISPR system can work in a synergic way to clarify if small-RNAs are positively or negatively related to the expression of their target genes, selectively turning off sRNAs with different expression between antibiotic resistant and sensitive strains, and then analyzing changes in the expression of their targets and possible phenotypic modifications.

Finally, these studies opened new ways and can be the starting point of further researches aiming to understand the role of trans-encoding small-RNA and CRISPR function in other pathogenic Staphylococci, as well as their usages in overcoming the antibiotic resistance.

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Tab.1 Phenotypic and molecular characteristics of the MRSA

Strain	Source	PFGE	MLST	SSC*mec*	Glycopeptide Heteroresistant Phenotype	MIC (mg/l)*			
						OXA	DAP	VAN	TEC
1-A	Skin infection	A1/ApaI	398	IVa	VSSA	32	<0.25	1	<0.25
1-C					hGISA	64	4	2	2
3-A	Blood culture	G1/SmaI	8	IV	hGISA	2	0.5	1	16
3-B					qVISA	16	4	2	2

*OXA (oxacillin) R > 2 mg/l;
DAP (daptomycin) R > 1 mg/l;
VAN (vancomycin) R > 2 mg/l;
TEC (teicoplanin) R > 2 mg/l

Tab.2 Paired-end (A) and Single-end (B) library Rockhopper results summaries

A

Paired-end	1-A	1-C	3-A	3-B
Total Reads	853091 (97%)	873327 (97%)	1010514 (98%)	101641 (97%)
Sense	50%	50%	49%	49%
Antisense	48%	48%	46%	46%
Unannotated	1%	1%	1%	1%
	1-A and 1-C		**3-A and 3-B**	
5'-UTR	51		99	
3'-UTR	3		70	
not antisense	8		11	
Antisense	19		22	
Differentially Expressed genes	341		210	
Likely operons	1057		1122	
Multigene operon	538		547	

B

Single-end	1-A	1-C	3-A	3-B
Total Reads	304494 (19%)	195956 (18%)	429270 (36%)	363244 (14%)
sense	80%	86%	53%	80%
Antisense	1%	0%	1%	0%
unannotated	5%	7%	7%	5%
	1-A and 1-C		**3-A and 3-B**	
5'-UTR	10		38	
3'-UTR	5		11	
not antisense	447		814	
antisense	882		1655	
Differentially Expressed genes	220		416	
Likely operons	1039		1069	
Multigene operon	533		544	

Tab.3 Coding-RNAs with differential expression between daptomycin resistant and sensitive isolates

						Expression (q-value ≤ 0,01)					
Regulation	***S. aureus* NCTC 8325 Locus Tag**	**Library[a]**	**Description**	**COG[b]**	**GO number[c]**	**1-C**	**1-A**	**q-value**	**3-B**	**3-A**	**q-value**
			Cell Wall and Cell Membrane Organization								
			Peptidoglycan Biosynthesis								
↓	SAOUHSC_02317	SE	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase MurF	M	GO:0009252	0	34	4,483E-4	0	39	1,11E-12
↓	SAOUHSC_00751	PE	Uncharacterized protein functionally related to murB	S	GO:0009987	0	53	3,56E-06	0	21	3,56E-06
↓	SAOUHSC_00022	SE	Regulatory protein YycH	S	-	0	49	0	0	52	0
			Cytolysis								
↑	SAOUHSC_02850	PE	Holin-like protein CidB	M	GO:0019835	49	0	0	277	54	1,94E-3
↓	SAOUHSC_00230	PE	Sensor histidine kinase LytS	T	GO:0071555	0	14	1,60E-19	0	21	0
			Cell Wall Division and Cell Membrane Structure								
↓	SAOUHSC_00486	PE	ATP-dependent zinc metalloprotease FtsH	O	GO:0006508	25	101	7,41E-3	54	422	8,53E-3
↑	SAOUHSC_03035	PE	Membrane spanning protein	S	-	21	0	0	40	0	0
↓	SAOUHSC_01919	SE	Integral membrane protein	-	-	0	95	0	0	463	0
↓	SAOUHSC_02376	SE	Membrane protein	S	-	0	221	0	0	143	1,57E-08
↓	SAOUHSC_02391	SE	Putative uncharacterized protein	-	-	0	3708	0	333	4282	6,07E-4
			Metabolism								
			Generation of Precursor Metabolites and Energy								
↑	SAOUHSC_02922	PE	L-lactate dehydrogenase 2 Ldh2	C	GO:0006096	28	0	0	42	1	1,04E-07
↓	SAOUHSC_01806	SE	Pyruvate kinase Pyk	G	GO:0006096	0	40	0	0	98	0
↓	SAOUHSC_01960	SE	Protoporphyrinogen oxidase	H	GO:0022904	0	44	5,81E-15	0	264	0
			Cofactor Metabolic Process								
↓	SAOUHSC_00984	PE	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase MenH	I	GO:0006732	0	35	0	0	124	1,32E-05
			Sulphur Compound Metabolic Process								
↓	SAOUHSC_01727	SE	Cysteine desulfurase	E	GO:0006790	0	56	0	0	57	0
			Nitrogen Compound Metabolic Processes								
↓	SAOUHSC_01774	SE	Porphobilinogen deaminase HemC	H	GO:0006778	0	95	0	0	138	0
			Phosphate-Containing Compounds Metabolic Process								
↓	SAOUHSC_02396	SE	Sugar phosphatase	R	GO:0006796	0	60	3,58E-07	0	70	0
↓	SAOUHSC_01055	SE	Inositol monophosphatase	G	GO:0006796	0	78	0	0	81	0

↓	SAOUHSC_00847	SE	ABC transporter	O	GO:0006796	0	86	0	0	72	4,95E-16
			Coenzyme Metabolic Process								
↓	SAOUHSC_00861	SE	Lipoyl synthase LipA	H	GO:0009107	0	69	0	0	93	9,17E-39
↓	SAOUHSC_02536	SE	Molybdenum cofactor biosynthesis protein A MoaA	H	GO:0006777	0	58	5,28E-13	0	96	4,97E-49
			Nucleotide Metabolic Process								
↓	SAOUHSC_00101	SE	Phosphopentomutase DeoB	G	GO:0009117	0	289	0	65	1256	6,75E-07
↓	SAOUHSC_01330	SE	Guanosine monophosphate reductase reductase GuaC	F	GO:0006163	0	210	0	0	262	0
↓	SAOUHSC_01169	PE	Carbamoyl-phosphate synthase small chain CarA	E, F	GO:0006221	0	26	1,12E-29	0	32	0
↓	SAOUHSC_01235	SE	Uridylate kinase PyrH	F	GO:0006221	0	79	0	0	74	1,11E-12
			Cellular Aminoacid Metabolic Process								
↓	SAOUHSC_01818	SE	Alanine dehydrogenase 2 Ald2	E	GO:0009063	0	56	0	0	132	0
			Lipid Metabolic Process								
↓	SAOUHSC_03006	SE	Lipase 1 LipA	R	GO:0016042	0	28	2,82E-14	0	27	1,88E-14
			Cellular Protein Modification Process								
↓	SAOUHSC_00741	SE	Ribonucleotide reductase stimulatory protein NrdI	F	GO:0006464	0	265	0	0	214	0
			Nucleic Acid Metabolic Process								
			DNA Repair								
↓	SAOUHSC_01693	SE	DNA-binding protein	L	GO:0006281	0	78	5,02E-08	0	59	6,93E-3
			DNA Replication								
↓	SAOUHSC_01690	SE	DNA polymerase III subunitδ	L	GO:0006260	0	126	0	0	46	3,86E-06
↓	SAOUHSC_02123	SE	ATP-dependent DNA helicase PcrA	L	GO:0006260	0	23	3,58E-07	0	44	0
			RNA Metabolic Process								
↓	SAOUHSC_01490	SE	DNA-binding protein HU	L	GO:1903506	0	249	1,75E-18	0	411	0
↓	SAOUHSC_01621	PE	Transcription antitermination protein NusB	K	GO:1903506	0	42	4,39E-06	0	126	0
↓	SAOUHSC_00031	SE	tRNA-dihydrouridine synthase	-	GO:0006399	0	220	0	0	168	0
↓	SAOUHSC_02116	SE	Aspartyl/glutamyl-tRNA amidotransferase subunit B GatB	J	GO:0006399	0	37	5,71E-08	0	70	0
↑	SAOUHSC_01455	SE	GTPase EngA	-	GO:0006399	45	0	0	11	0	0
↓	SAOUHSC_01188	SE	Ribosome biogenesis GTPase RsgA	R	GO:0042254	0	56	1,57E-05	0	98	9,17E-39
			Response to Stress								
↑	SAOUHSC_01334	PE	Presumptive SOS response gene functionally related to LexA	-	-	61	0	0	134	0	0
↓	SAOUHSC_01682	SE	Chaperone protein DnaJ	O	GO:0009408	16	144	9,67E-05	0	180	0
↑	SAOUHSC_00304	SE	Monooxygenase	C	GO:0006979	63	0	2,47E-12	57	0	0
↑	SAOUHSC_00406	SE	PHB depolymerase	-	GO:0042594	53	0	0	28	0	0
↓	SAOUHSC_00204	SE	Globin domain containing protein - Nitric oxide dioxygenase	C	GO:0071500	0	55	0	0	35	6,93E-3
			Transport								

↓	SAOUHSC_00099	SE	Transporter	G	GO:0055085	0	90	0	0	370	0
↓	SAOUHSC_00137	PE	Transporter	P	GO:0006810	0	26	0	0	48	0
↓	SAOUHSC_02700	PE	Transporter	-	GO:0055085	0	19	0	0	32	0
↓	SAOUHSC_00888	PE	Na+/H+ antiporter subunit B1 MnhB1	P	GO:0006812	0	115	0	0	40	0
↓	SAOUHSC_00634	SE	ABC transporter	P	GO:0030001	0	103	0	0	96	0
↑	SAOUHSC_01387	PE	Inorganic phosphate transmembrane transporter	P	GO:0006817	68	0	0	48	2	1,55E-07
			Carbohydrate Transport								
↓	SAOUHSC_00177	SE	Maltose ABC transporter permease	G	GO:0008643	0	45	0	0	142	0
↓	SAOUHSC_02400	SE	PTS system, mannitol-specific component	G	GO:0008643	0	40	5,81E-15	0	66	0
↓	SAOUHSC_00158	SE	PTS system N-acetylmuramic acid-specific component	G	GO:0008643	0	33	1,57E-05	0	73	0
			Cell Adhesion								
↓	SAOUHSC_00545	SE	Serine-aspartate repeat-containing protein D - SdrD	-	GO:0007155	0	99	0	0	30	0
			Unknown								
↑	SAOUHSC_00826	PE	Conserved uncharacterized protein	S	-	38	0	0	19	0	1,626E-05

↑ up-regulated mRNAs in DAP-R *vs* DAP-S
↓ down-regulated mRNAs in DAP-R *vs* DAP-S
a - SE=Single-end; PE=Paired-end
b – COG categories:: C= energy production; E= Amino Acid metabolism and transport, F= Nucleotide metabolism and transport; G=carbohydrate metabolism and transport; H= Coenzyme metabolism; I=lipid metabolism; J= Tranlsation; K= Transcription; L= Replication and repair; M= Cell wall/membrane/envelop biogenesis; O= Post-translational modification, protein turnover, chaperone functions; P= Inorganic ion transport and metabolism; Q=secondary structure; R=general functional prediction only; S=function unknown; T= Signal Transduction
c – Gene Ontology numbers refer only to the biological process

Tab.4 Non-coding RNAs with differential expression between daptomycin resistant and sensitive isolates

						Expression (*q*-value ≤ 0,01)						
Regulation	***S. aureus* NCTC 8325 Locus Tag**	**Description**	**COG[a]**	**GO number[b]**	**sRNA size 1C/1A**	**1-C**	**1-A**	***q*Value**	**3-B**	**3-A**	***q*Value**	**sRNA size 3B/3A**
		CELL-WALL AND CELL MEMBRANE ORGANIZATION										
		Peptidoglycan Biosynthesis										
↑	SAOUHSC_02337	UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA	M	GO:0009252	325 bp	294	0	0	1250	0	0,000234	22 bp
		METABOLISM										
		Aminosugar Biosynthesis										
↓	SAOUHSC_02423	Putative UDP-N-acetylglucosaminepyrophosphorylase	G	GO:0008152	50 bp	0	2081	5,03E-08	0	1935	1,58E-08	25 bp
		Cathecols biosynthesis										
↓	SAOUHSC_00906	Cathecols biosynthesis	Q	GO:0008152	38 bp	0	1628	0	0	1974	0	45 bp
		Tricarboxylic Acid Cycle										
↑	SAOUHSC_01418	2-oxoglutarate dehydrogenase OdhA	C	GO:0006099	28 bp	2190	0	2,48E-12	1250	0	0	46 bp
		Nucleotide Metabolic Process										
↓	SAOUHSC_01330	Guanosine monophosphate reductase GuaC	F	GO:0006163	43 bp	0	3743	0	0	1931	0,000753	21 bp
		Coenzyme Metabolic Process										
↓	SAOUHSC_02543	Molybdenum cofactor biosynthesis protein C MoaC	H	GO:0006777	24 bp	0	2870	0	0	1802	8,34E-16	32 bp
↓	SAOUHSC_00500	Pyridoxal 5'-phosphate synthase subunit PdxT	H	GO:0009110	25 bp	0	2081	5,03E-08	0	2389	0	31 bp
		Lipid Metabolic Process										
↓	SAOUHSC_02860	HMG-CoA synthase MvaS	I	GO:0008299	27 bp	0	2352	0	0	1479	0	56 bp
		Cellular Aminoacid Metabolic Process										
↑	SAOUHSC_02610	Formimidoylglutamase HutG	E	GO:0006547	84 bp	1255	0	6,98E-40	2500	0	0	22 bp
		NUCLEIC ACID METABOLIC PROCESS										
		DNA Replication										
↓	SAOUHSC_00349	Single-stranded DNA binding protein	L	GO:0006260	123 bp	0	1309	0	0	3322	0	465 bp
		DNA repair										
↓	SAOUHSC_01333	LexA repressor	K, T	GO:0006281	102 bp	0	616	0	0	1307	0,002428	31 bp
		RNA Metabolic Process										
↓	SAOUHSC_01035	Ribonuclease J1 Rnj1	R	GO:0006364	24 bp	0	6823	0	0	9443	0	28 bp
↓	SAOUHSC_00951	Putative phosphoesterase	J	-	23 bp	0	3529	0	0	1844	4,04E-11	27 bp
		RIBOSOMAL RNA										
↓	SAOUHSC_R00016	5S Ribosomal RNA	-	-	65 bp	0	730	7,16E-05	0	987	0	140 bp
↓	SAOUHSC_R0001	16S Ribosomal RNA	-	-	53 bp	0	2309	0	0	1650	0	134 bp
↓	SAOUHSC_R0002	16S Ribosomal RNA	-	-	56 bp	0	3137	0	0	1056	0	127 bp
↓	SAOUHSC_R0003	16S Ribosomal RNA	-	-	84 bp	0	622	5,71E08	0	565	0	163 bp
↓	SAOUHSC_R0005	16S Ribosomal RNA	-	-	50 bp	0	1199	0	0	3289	0	30 bp
↓	SAOUHSC_R0006	23S Ribosomal RNA	-	-	81 bp	0	1592	0	0	2552	0	295 bp
↓	SAOUHSC_R0007	23S Ribosomal RNA	-	-	78 bp	0	2635	0	0	3810	0	93 bp
↓	SAOUHSC_R0008	23S Ribosomal RNA	-	-	89 bp	0	2483	0	0	1116	0	102 bp
↓	SAOUHSC_R0009	23S Ribosomal RNA	-	-	19 bp	0	3529	0	55	1158	4,07E-11	625 bp
↓	SAOUHSC_R00010	23S Ribosomal RNA	-	-	81 bp	0	659	5,03E-08	0	411	2,91E-05	107 bp
		TRANSFER RNA										
↓	SAOUHSC_T00059	tRNA-Val	-	-	65 bp	0	730	7,16E-05	0	987	0	140 bp
		RESPONSE TO STRESS										
↓	SAOUHSC_00093	Superoxide dismutase SodM	P	GO:0019430	29 bp	0	2352	0	0	1055	0	51 bp
↓	SAOUHSC_00912	ATP-dependent Clp protease; ATP-binding subunit ClpB	O	GO:0009408	19 bp	0	2352	0,000449	0	829	0,000266	51 bp
↓	SAOUHSC_01431	Putative methionine sulfoxide reductase B MsrB	O	GO:0006979	25 bp	0	2307	5,28E-13	0	3921	0	25 bp
↓	SAOUHSC_03045	Cold Shock-like Protein CSPF	K	GO:0006355	28 bp	0	3123	0	0	774	0	23 bp

		TRANSPORT										
↑	SAOUHSC_00637	Hypothetical protein (Membrane Transporter)	P	GO:0006865	27 bp	2557	0	0	823	0	0	87 bp
↑	SAOUHSC_01354	Sodium ion transport alanine transporter	E	GO:0006814	20 bp	2702	0	1.56E-06	1250	0	1,23E-06	24 bp
↓	SAOUHSC_00738	Peptide transporter	E	GO:0006857	24 bp	0	2352	2,82E-14	0	1960	0	31 bp
		UNKNOWN FUNCTION										
↑	SAOUHSC_02853	Hypothetical protein	-	-	27 bp	2702	0	0	1250	0	0	27 bp
↓	SAOUHSC_00452	Hypothetical protein	S	-	21 bp	0	2352	3,58E-07	0	923	0	113 bp
↓	SAOUHSC_00907	Hypothetical protein	-	-	80 bp	0	2527	0	0	1532	0	54 bp

↑ up-regulated mRNAs in DAP-R *vs* DAP-S
↓ down-regulated mRNAs in DAP-R *vs* DAP-S
a – COG categories:: C= energy production; E= Amino Acid metabolism and transport, F= Nucleotide metabolism and transport; G=carbohydrate metabolism and transport; H= Coenzyme metabolism; I=lipid metabolism; J= Tranlsation; K= Transcription; L= Replication and repair; M= Cell wall/membrane/envelop biogenesis; O= Post-translational modification, protein turnover, chaperone functions; P= Inorganic ion transport and metabolism; Q=secondary structure; R=general functional prediction only; S=function unknown; T= Signal Transduction
b – Gene Ontology numbers refer only to the biological process

Tab.5 Italian pathogenic MDR *S. epidermidis* phenotypic and molecular characteristics

								ANTIBIOTYPE*				
Strain	**Source**	**PFGE**	**MLST**	***erm*B**	***erm*A**	***erm*C**	***cfr* plasmid**	**CAM**	**ERY**	**MLS**	**LZD**	**TEC**
IS1	Central venous catheter	C1	2	NEG	NEG	NEG	+	R	R	R	R	S
IS2	Central venous catheter	C1	2	NEG	NEG	NEG	+	R	S	R	R	S
IS3	Blood culture	A1	23	NEG	POS	NEG	+	R	R	R	R	R
IS4	Blood culture	A1	23	NEG	POS	NEG	-	R	R	R	R	R
IS5	Blood culture	A1	23	NEG	POS	NEG	-	R	R	R	R	R
IS6	Blood culture	A2	23	NEG	POS	NEG	+	R	R	R	R	S
IS7	Peritoneal fluid	A2	23	NEG	NEG	POS	+	R	R	R	R	R
IS8	Blood culture	A2	/	/	/	/	-	S	R	R	R	S
IS9	/	A4	/	/	/	/	+	R	R	R	R	R
IS10	/	F	5	/	/	/	+	R	R	R	R	R

*CAM: chloramphenicol; ERY: erythromycin; MLS: macrolides/lincosamides/streptogramin; LZD: linezolid; TEC: teicoplanin

Tab.6 Primers used in the study to detect CRISPR-Cas genes

Gene or region	Primer	Sequence (5' → 3')	Product length (bp)	Annealing temperature	Reference Strain
cfr	cfr-fw	TGA AGT ATA AAG CAG GTT GGG AGT CA	746	53 °C	[138]
	cfr-rev	ACC ATA TAA TTG ACC ACA AGC AGC			
*cas*1	SS001	AAT CAT TAC TTT GTT ACT GCG AAG GAA A	344	53 °C	ATCC 35984
	SS002	TTT CAA GAC ATC GTG TTT GGT TAA A			
*cas*2	SS003	TTA ATA GAA CTT GGA TTT AGT ATG AAG C	212	53 °C	ATCC 35984
	SS004	TTT GGT TCT TTG TTG ACA GCA			
1st R/S-*cas*1	FW057	TAT TTT TTG ACA GCA AAA ATG ATG CTT GAA ATA TAG	Depends on the strain	55 °C	This study
	AA406	AAA TTT AAT GCT ATT TTC CTT CGC			
*cas*1-*cas*2	FW290	TAA ATC TAA CAA CAC TCT AAA AAA TTG TAG ATT TTG	1267	55 °C	RP62a
	FW207	GGT TCT TTG TTG ACA GCA AGC			
*cas*1-*cas*10	AA422	TTA TGG TTA TTC AAT TCT CAG ATC	2300	55 °C	RP62a
	FW066	GCA CCG AGA TTA TCT ATA TCG GCA CGT ACC ACG			
*cas*10	AA423	ATC AAT TTT TGT CCC AAT TTT CAG	1501	55 °C	RP62a
	FW066	GCA CCG AGA TTA TCT ATA TCG GCA CGT ACC ACG			
*cas*10-*csm*2	AA425	CAA ATT ACTG CTA TAT ATT CAG GC	936	55 °C	RP62a
	FW012	CTA AGA AAA ATT TTT TTG ATT CTT TTT TTA TCA CTC TAT C			
*csm*2-*csm*4	AA426	TTA AAT TTT ATT ATG AAG CAG GAC G	908	55 °C	RP62a
	FW010	CAC CAT CTG ACA ACC GTT TTT TTC C			
*csm*3-*csm*5	FW005	TCA CTT ATT AGA GAA TGC CTA TCT TGG TGG	1067	55 °C	RP62a
	FW085	GTG GTG GTG GTG GTG CTC GAG TTA TAC CTC CAA CCA TAA AGG TTT TGC			
*csm*4-*csm*5	AA415	ACA AGA AAC TGA TTC AAG TGC TG	1058	55 °C	RP62a
	FW133	CTT TGT TCA ATT TCA TTA ATA GAA TAA ATT GCA TCT TCA ATT GTT AAC			
*csm*5-*csm*6	AA416	TAT TCT GAA AAG GTC AAT CAA GG	1171	55 °C	RP62a
	FW113	CTA TCC TTA GTT GGC GTG CTC A			
*csm*6-*cas*6	AA417	GCG ATG CTT CAT ATC GTG CG	1361	55 °C	RP62a
	FW115	TCT TTG TTT CAA TGG GCT ATA AGC A			
*csm*6-*cas*6	AA418	CTA CTT TAA TAA TTG AAA AAG ATG G	1191	55 °C	RP62a
	AA101	CAC CTT TAC TAT AGA CGC TAA ATG TCA C			
*cas*6	FW114	TAG GAA GTG TTT TAC ATG GTG TGT	739	55 °C	RP62a
	AA101	CAC CTT TAC TAT AGA CGC TAA ATG TCA C			
*cas*6-2nd R/S	AA419	GTC TTT TAA ATA TCA GAA CAG TTA C	Depends on the strain	55 °C	This study
	AA457	CTC ATG TCT TGA GAA CTA GGA ATA CC			

Tab.7 CRISPR-Cas locus architecture of the Italian *S. epidermidis* strains

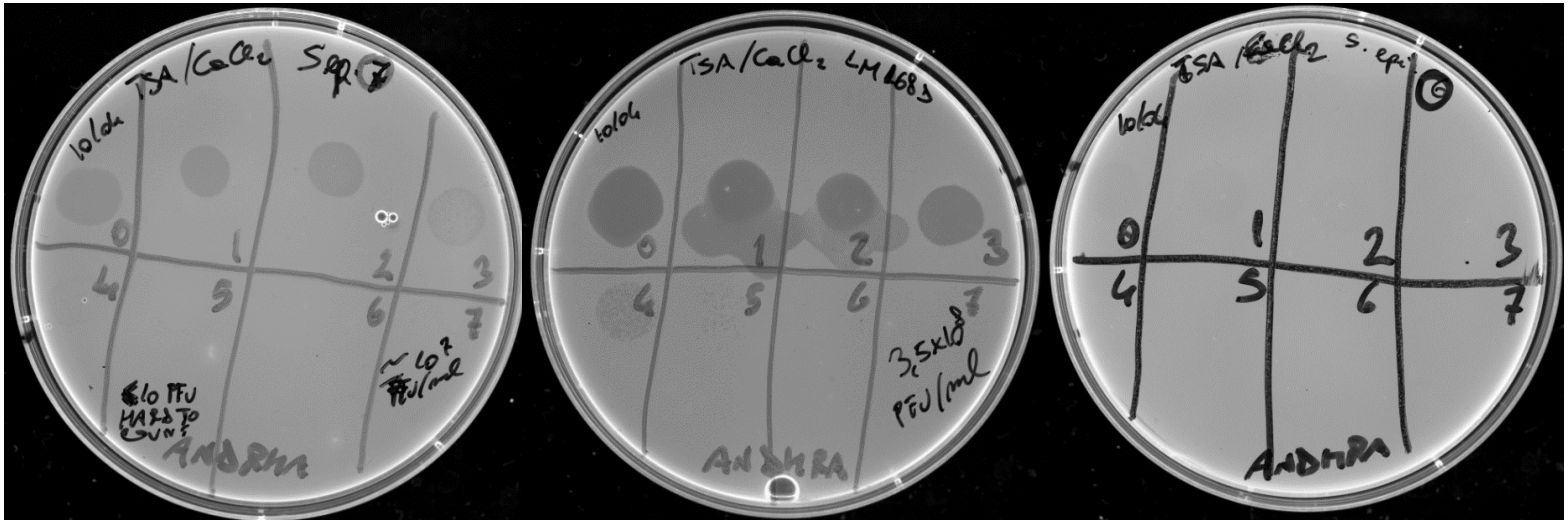
Strain	1st R/S	*cas*1	*cas2*	*cas*10	*csm2*	*csm3*	*csm4*	*csm5*	*csm6*	*cas6*	2nd R/S
IS1	nt*	-	-	-	nt	nt	nt	nt	nt	+	-
IS2	nt	-	-	-	nt	nt	nt	nt	nt	+	-
IS3	nt	-	-	+	+	+	+	+	+	+	-
IS4	nt	-	-	+	+	+	+	+	+	+	-
IS5	nt	-	-	-	nt	nt	nt	nt	nt	+	-
IS6	9	+	+	+	+	+	+	+	+	+	2
IS7	11	+	+	+	+	+	+	+	+	+	2
IS8	9	+	+	+	+	+	+	+	+	+	2
IS9	nt	-	-	-	nt	nt	nt	nt	nt	+	-
IS10	nt	+	+	-	nt	nt	nt	nt	nt	+	-

*nt: not tested

Tab.8 List of SNPs in the IS7 CRISPR-Cas genes

Gene	SNPs	Amino Acid Substitution
*cas*1	G180A	/
	A331G	I111V
	C361T	L121F
	A457G	T153A
*cas*2	C204T	/
*cas*10	C125T	/
	G192T	A64S
	C233T	/
	T422G	/
	G906A	A303T
	T921C	/
	G972T	V325L
	T1127C	/
	C1133T	/
	T1175C	/
	G1214A	/
	A1319C	/
	A6210T	/
	A6126T	/
	T1865A	/
	C1886T	/
	A1916G	/
	G2045A	/
	G2069A	/
	T2138C	/
	G2177A	/
*csm*2	G112A	V46I
	A169C	/
*csm*3	A170G	N57S
	C351T	/
	C405T	/
	T449A	F150Y
	G480A	/
*csm*4	T2A	M1K
	G714A	/
	G748A	V250I
	A835G	N279D
*csm*5	C24T	/
	A28G	I10V
	A46G	I16V
	T169C	Y57H
*csm*5	G192A	/
	C297T	/
	T306C	/
	AG322GT	T108V
	T330A	/
	T342C	/
	C351T	/
	T408C	/
	C426T	/
	C453T	/
	C459T	/
	T498C	/
	T553C	S185P
	T562C	/
	A577G	I193V
	C591T	/
	A651G	/
	AA659GC	Q220R
	C669T	/
	C696T	/
	A699T	/
	A882T	/
*csm*6	A7G	I3V
	C330T	/
	G403A	V135I
	C642A	/
	A775T	N259Y
	T794A	L265H
	GT896AC	G299D
	C907T	/
	T937G	Y313D
	T948G	N316K
	G951A	/
	A959G	E320G
	A969C	K323N
	T975C	/
	C1011T	/
	T1014A	/
	C1045A	Q349K
	G1234A	E412K
	C1261T	/
*cas*6	C30T	/
	G33A	/
	A39T	E13D
	G41A	S14N
	G48A	/
	G67A	V23I
	G81A	/
	A96T	/
	T97C	S33P
	G100A	D34N
	T132C	/
	C141T	/
	C163T	H55Y
	A170G	N57S
	ATC178GTA	I60V
	A219G	/
	G223A	V75I
	T303C	/
	G327T	M109I
	G339T	/
	A342G	/
	GT346AC	V116T
	G359A	S120N
	A381T	/
	A384G	/
	AC410GT	N137S
	C471T	/
	G474T	/
	G491A	R164K
	A514G	N172D
	C520T	/
	GAA523ATG	E175M
	G528A	/
	T543G	/
	G576A	/
	A597G	/
	A612G	I204M
	A615G	/
	A630C	/
	C643T	/
	A696G	I232M

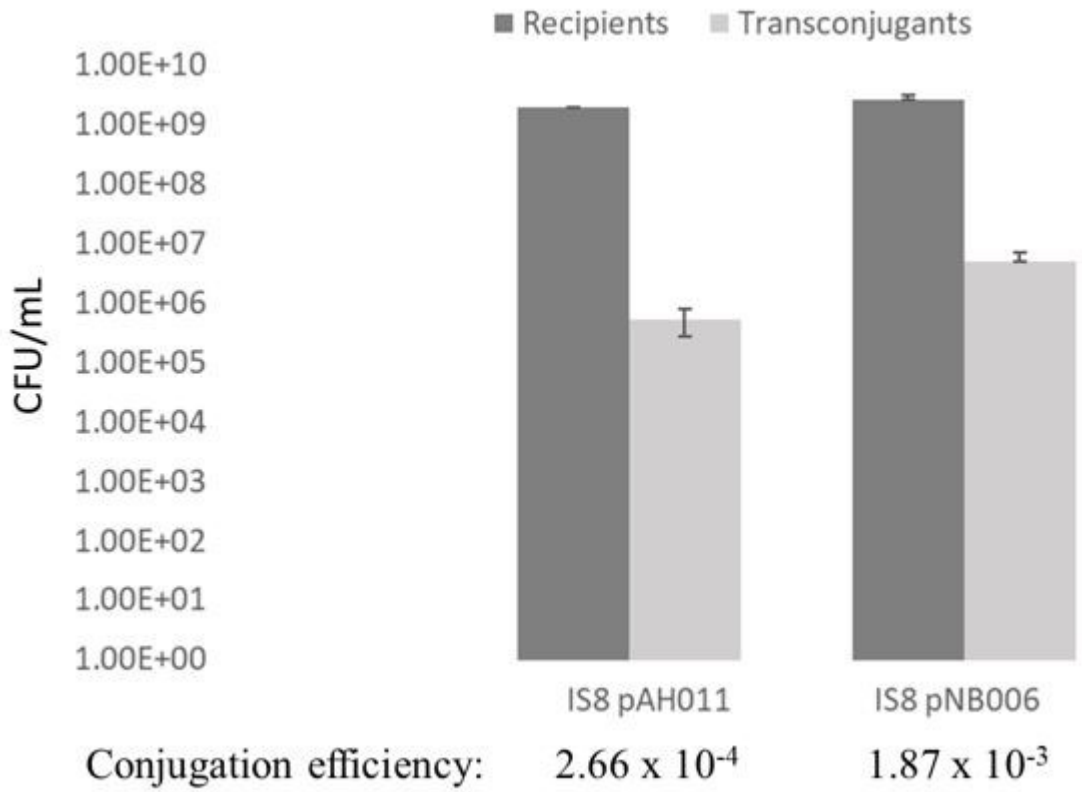
Fig.1 IS7 infection with Andhra phage and phage infection positive and negative controls



Tab.9 Phage infection sensitivity of the Italian *S. epidermidis*

Strain	Phage		
	ANDHRA	**CNPx**	**ISP**
IS1	No Plaques	No Plaques	4×10^9 PFU/mL
IS2	No Plaques	No Plaques	No Plaques
IS3	No Plaques	No Plaques	No Plaques
IS4	No Plaques	No Plaques	No Plaques
IS5	No Plaques	No Plaques	No Plaques
IS6	No Plaques	No Plaques	No Plaques
IS7	2×10^7 PFU/ml	No Plaques	No Plaques
IS8	No Plaques	No Plaques	No Plaques
IS9	No Plaques	No Plaques	4×10^7 PFU/mL
IS10	No Plaques	No Plaques	No Plaques

Fig.2 Anti-plasmid activity of the IS8 CRISPR-Cas system



IS8 pNB006: positive control of conjugation

Main abbreviations and acronyms in alphabetical order

CAM: chloramphenicol

CFU: colony forming unit

COGs: clusters of orthologous groups

CoNS: coagulase-negative Staphylococci

CoPS: coagulase-positive Staphylococci

CRISPR: clustered regularly interspaced short palindromic repeats

DAP: daptomycin

dH_20: distilled water

EDTA: ethylenediaminetetraacetic acid

ERY: erythromycin

GISA: glycopeptide-intermediate *Staphylococcus aureus*

GO: gene ontology

GRSE: glycopeptide-resistant *Staphylococcus epidermidis*

hVISA/VISA: heterogeneous vancomycin-intermediate / vancomycin-intermediate *Staphylococcus aureus*

KEGG: Kyoto encyclopedia of genes and genomes

LZD: linezolid

MIC: minimum inhibitory concentration

MLS: macrolides/lincosamides/streptogramin

MOI: multiple of infection

MRSA: methicillin-resistant *Staphylococcus aureus*

MRSE: methicillin-resistant *Staphylococcus epidermidis*

MSSA: methicillin-susceptible *Staphylococcus aureus*

NGS: next generation sequencing

OD: optical density

OXA: oxacillin

PANTHER: protein analysis through evolutionary relationships

PFU: plaque forming unit

RPMK: reads per kilobase million

SDS: sodium dodecyl sulphate

SSC: Staphylococcal Cassette Chromosome

STRING: search tool for the retrieval of interacting genes/proteins

TEC: teicoplanin

VAN: vancomycin

VISE: vancomycin-intermediate *Staphylococcus epidermidis*

VSSA: vancomycin-susceptible *Staphylococcus aureus*

WHO: world health organization