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ROLE HORIZONTÁLNÍHO PŘENOSU GENŮ V EVOLUCI RODU *STAPHYLOCOCCUS*

Diplomová práce

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Abstrakt

Stafylokoky jsou úspěšnými komenzály či patogeny především díky tomu, že se velmi rychle adaptují na změny prostředí. V takovémto adaptačním procesu hraje hlavní úlohu horizontální přenos genů, který umožňuje i nepříbuzným druhům bakterií vyměňovat genetickou informaci. Často jsou vyměňovány mobilní genetické elementy (MGE) kódující virulenční faktory či molekuly zodpovědné za rezistenci k antibiotikům. Tato práce je zaměřena na MGE koaguláza-negativních stafylokoků (KNS), především druhů *Staphylococcus sciuri* a *Staphylococcus petrasii*, a na možný přenos MGE transdukcí. Byly objeveny čtyři nové temperované bakteriofágy infikující KNS za použití nově navržené skríningové metody založené na UV-reaktivaci profágů. Transdukční schopnosti těchto fágů nebyly prokázány, přestože relativní kvantifikace prokázala přítomnost bakteriálních genů ve fágových kapsidách. Dále byl analyzován obsah plazmidů u výše zmíněných druhů a jeden plazmid *S. sciuri* byl osekvenován a anotován. Také byla anotována sekvence nové stafylokokové kazety *mec* (SCC*mec*) typu VII z genomu *S. sciuri*. Tyto výsledky společně s fylogenetickou studií 81 stafylokokových chromozomových kazet (SCC), přispívají k názoru, že KNS jsou zásobárnou i místem rekombinace pro MGE stafylokoků.

Abstract

Staphylococci are successful as commensals or pathogens in part they adapt rapidly to changes in the environment. The horizontal gene transfer plays the central role in this adaptation process enabling unrelated bacteria to exchange genetic information. Frequently exchanged mobile genetic elements (MGEs) encode often putative virulence factors and molecules that confer resistance to antibiotics. This work focuses on the MGEs in coagulase negative staphylococci (CoNS), namely *Staphylococcus sciuri* and *Staphylococcus petrasii*, and their possible transmission via transduction. Four new temperate bacteriophages of CoNS were discovered using new screening method based on the reactivation of the prophages by UV-light. Transduction ability of the two discovered phages was not confirmed, though relative quantification proved that parts of bacterial MGEs are packaged inside phage capsids. Combined with analysis of plasmid content, including sequencing of one plasmid from *S. sciuri*, identification of new staphylococcal cassette chromosome *mec* (SCC*mec*) type VII from genome sequencing and the phylogenetic study of 81 staphylococcal cassette chromosome (SCC) elements, the results indicate that CoNS could serve as a reservoir and recombination hotspot for staphylococcal MGEs.

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Cílem práce bude popis horizontálního přenosu genů především u druhu *Staphylococcus sciuri*. Práce bude zahrnovat indukci bakteriofágů u vytipovaných druhů *S. sciuri*, návrh a optimalizaci transdukčních experimentů, selekci a analýzu transduktant prostřednictvím molekulárně-biologických metod. U transdukujících fágů bude provedena kvantifikace horizontálně přenášených genů pomocí qPCR. Součástí práce bude i fylogenetické srovnání SCC-elementů.

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Prohlašuji, že jsem svou diplomovou práci vypracovala samostatně s využitím informačních zdrojů, které jsou v práci uvedeny.

Brno 9. května 2016 **…….**

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List of abbreviations

ACME	Arginine Catabolic Mobile Element
AIC	Akaike information criterion
BLAST	Basic local alignment search tool
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CCM	Czech collection of microorganisms
CDS	Coding sequence
CFU	Colony forming unit
CoNS	Coagulase-negative staphylococci
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
Inc	Incompatibility
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LCB	Locally collinear block
LMDM	Laboratory of molecular diagnostics of microorganism
LPSN	List of prokaryotic names with standing in nomenclature
MGE	Mobile genetic elements
MLST	Multilocus sequence type
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Modification and specifity
NCBI	National center for biotechnology information
NRL/St	National reference laboratory for staphylococci
ORF	Open reading frame
PBP	Penicillin-binding proteins
PFGE	Pulse-field gel electrophoresis
PFU	Plaque forming units
PRSA	Penicillin-resistant *Staphylococcus aureus*
RAST	Rapid annotations using subsystems technology
RAxML	Randomized axelerated maximum likelihood
RFLP	Restriction fragments length polymorphism
RM	Restriction-modification
SaPI	Staphylococcal pathogenicity island
SCC	Staphylococcal cassette chromosome
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCC*mer*	Staphylococcal cassette chromosome *mercury*
ST	Sequence type

1 Introduction

Staphylococci are major human and veterinary pathogens causing local and systemic infections. The pathogenicity of these bacteria depends on the production of virulence factors and content of antimicrobial resistance determinants (Lindsay, 2014). Genes for the virulence and resistance factors are carried on different types of mobile genetic elements (MGEs), such as genomic islands, pathogenicity islands, prophages, plasmids, and staphylococcal cassette chromosome (SCC), which can be horizontally transferred. Horizontal gene transfer (HGT) is responsible for the exchange of beneficial traits which enable bacteria to establish a new lifestyle that can be accompanied by an adaptation to a new ecological niche or an increase in pathogenicity.

The most thoroughly studied pathogenic bacterium of the genus *Staphylococcus* is undoubtedly *Staphylococcus aureus*. In recent years, other species of this genus got into the focus of scientists either for their pathogenic characteristics or for their potential to serve as a reservoir of virulence or resistance genes.

This work focuses on the MGEs and their role in the evolution of the genus *Staphylococcus*. Particular emphasis is placed on the coagulase-negative staphylococci (CoNS), namely the *Staphylococcus sciuri* species, which might serve as a source of methicillin resistance (Tsubakishita *et al.*, 2010b), and the newly described related species *Staphylococcus petrasii*.

2 Overview of genus *Staphylococcus*

Staphylococci were first described by Pasteur and Koch. In 1880, a Scottish surgeon Alexander Ogston discovered the major cause of pus and named the pathogenic bacteria staphylococci. Later, in 1884, a German surgeon Anton J. Rosenbach isolated two pigmented colony types of staphylococci and proposed the nomenclature: *S. aureus* (yellow) and *Staphylococcus albus* (white), which is now called *Staphylococcus epidermidis*. Staphylococci are round-shaped Gram-positive bacteria that form grape-like clusters (Ryan and Ray, 2004). Taxonomically, the genus *Staphylococcus* is in the family *Staphylococcaceae*, which includes three other genera, *Gamella*, *Macrococcus* and *Salinicoccus*. The family *Staphylococcaceae* belongs to order *Bacillales*, class *Bacilli*, phylum *Firmicutes*, and kingdom *Bacteria* (Schleifer and Bell, 2009). The genus *Staphylococcus* consists of 52 species with 28 subspecies according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) website (Euzeby, 1997).

Staphylococci are catalase-positive, stationary, non-sporulating bacteria and mostly facultative anaerobes. Unlike other bacteria, they are able to grow in the presence of 10 % NaCl (Ryan and Ray, 2004). Most species of this genus are harmless, they are part of soil microbial flora or more often they reside on skin and mucous membranes of animals including humans. The majority of the species in this genus does not produce coagulase, an enzyme that causes blood clot formation. However, the most clinically significant species *S. aureus* is coagulase-positive. This thesis deals with *S. aureus* and CoNS, such as *S. sciuri* and *S. petrasii*, and their contribution to the evolution of pathogenic staphylococci.

S. aureus is a ubiquitous microorganism that colonises asymptomatically 20–50 % human population (von Eiff *et al.*, 2004). *S. aureus* is also an important hospital and community-acquired pathogen. It is often responsible for wound infections, post-surgical infections, fatal pneumonia, endocarditis, and meningitis. In healthy individuals it is usually found on skin and mucous membranes (Becker *et al.*, 2003). Colonization by *S. aureus* predisposes its carriers to higher risk of infection and it is an important source for the dissemination of *S. aureus* in the community (Chambers and Deleo, 2009). The emergence and spread of antibiotic-resistant strains poses a serious threat in health-care, especially the strains of methicillin-resistant *S. aureus* (MRSA).

CoNS are common commensals of mammals isolated mainly from the mammals' skin, mucous membranes, glands, milk, and from blood cultures. CoNS have long been regarded as

non-pathogenic, but in the last three decades the role of CoNS in human infections is being recognized (Huebner and Goldmann, 1999). They are opportunistic pathogens causing mild to fatal infections especially in patients with indwelling devices or immunocompromised individuals and a leading cause of otic infections (Roland and Stroman, 2002).

S. sciuri is a novobiocin-resistant CoNS, first isolated from the fur of *Sciurus carolinensis* (Kloos *et al.*, 1976). The *S. sciuri* species group includes five species: *S. sciuri* (with three subspecies), *Staphylococcus lentus*, *Staphylococcus vitulinus*, *Staphylococcus fleurettii* and *Staphylococcus stepanovicii*. These bacteria are commonly found in a broad range of habitats including animals, humans and the environment (Nemeghaire *et al.*, 2014). At first, it was considered to be non-pathogenic, as *S. sciuri* species are found on the skin of animals and on mucous membranes (Bagcigil *et al.*, 2007). However, nowadays it is correlated with some animal diseases such as dog dermatitis (Hauschild and Wojcik, 2007), exudative epidermitis in pigs (Chen *et al.*, 2007) and with growing number of nosocomial diseases of people, namely endocarditis, pelvic inflammation and wound infection (Hedin and Widerstrom, 1998; Kolawole and Shittu, 1997; Stepanovic *et al.*, 2002; Stepanovic *et al.*, 2005).

S. petrasii was first described as a new species in 2013 in the Czech Republic (Pantucek *et al.*, 2013). This species is named in honor of Dr. Petr Petráš, a Czech microbiologist, for his contribution to the taxonomy of staphylococci. *S. petrasii* is related to *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus devriesei*, and *Staphylococcus lugdunensis* according to the phylogenetic analysis based on 16S rRNA (Pantucek *et al.*, 2013). The species is currently divided into four subspecies - *Staphylococcus petrasii* subsp. *petrasii*, and *Staphylococcus petrasii* subsp. *croceilyticus, Staphylococcus petrasii* subsp. *jettensis*, and *Staphylococcus petrasii* subsp. *pragensis* (De Bel *et al.*, 2014). *S. petrasii* is likely to be an opportunistic pathogen as other CoNS, because strains of this species were identified in human clinical material.

2.1 Emergence of antibiotic resistance in staphylococci

Staphylococci acquire resistance to therapeutics shortly after their introduction in the clinical usage. Mainly clones of *S. aureus* are exhibiting disturbing potential to accumulate pathogenic characteristics as a result of HGT due to selective pressure caused by the overuse of antimicrobials in health care environment. New antibiotic-resistant strains of *S. aureus* appear shortly after the introduction of a new therapeutic agent as illustrated in Figure 1.

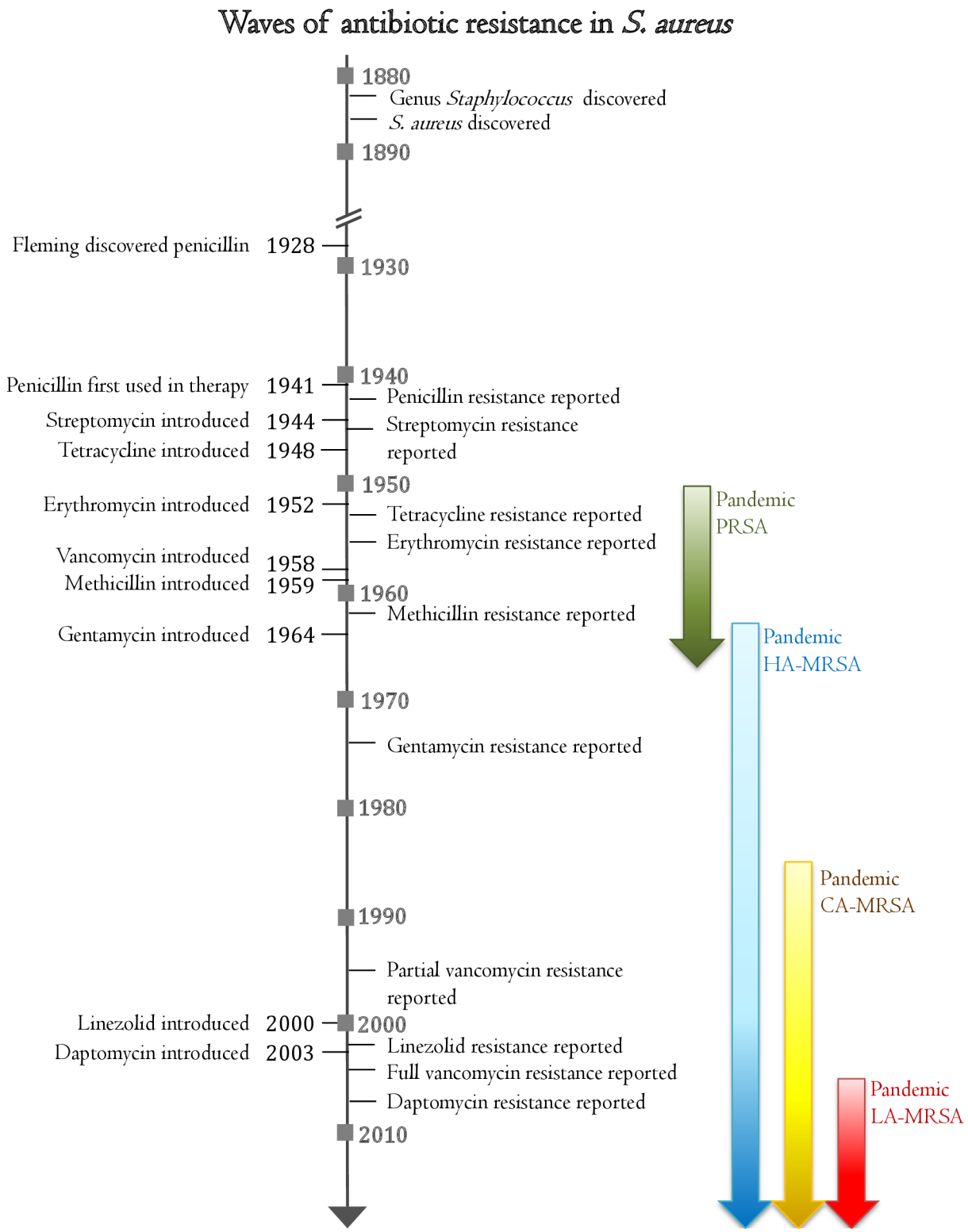

Figure 1: Timeline showing occurrence of antibiotic-resistant *S. aureus* and epidemic waves of this pathogen. PRSA – penicillin-resistant *S. aureus*, HA-MRSA – hospital-acquired methicillin-resistant *S. aureus*, CA-MRSA – community-acquired MRSA, LA-MRSA, livestock-associated MRSA.

In bacteria, antibiotic resistance is facilitated via three main mechanisms:

1. Inactivation of the antibiotic as observed with resistance to β-lactam and aminoglycoside antibiotics.

2. Efflux of the antibiotic from the cell as observed with resistance to ciprofloxacin, erythromycin and tetracycline.

3. Target modification as observed with resistance to ciprofloxacin, erythromycin, fusidic acid, mupirocin, rifampicin, and tetracycline (McCallum *et al.*, 2010).

When a lineage of *S. aureus* gains drug resistance, it can become epidemic as is the case of penicillin-resistant *S. aureus* (PRSA), hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA), community-acquired MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA). To stop the spread of antibiotic-resistant clones, several actions have been implemented, such as worldwide monitoring of the most feared methicillin resistance incidence in staphylococci.

2.2 Methicillin resistance in staphylococci

Methicillin is a semi-synthetic, β-lactam antibiotic and it was first developed in 1959 to treat infections caused by penicillin-resistant *S. aureus* (Stewart and Holt, 1963). Penicillin resistance is conferred by β-lactamase, which hydrolyzes the β-lactam ring of the penicillin, thereby inactivating the antibiotic. β-lactamase is encoded by the *blaZ* gene in staphylococci (Olsen *et al.*, 2006). The structure of methicillin is different from benzylpenicillin. Methicillin has added methoxy groups to the phenol group, which produces a steric hindrance around the amide bond and reduces the affinity for staphylococcal β-lactamases (Stapleton and Taylor, 2002). Methicillin and other β-lactam antibiotics inhibit the cell wall synthesis by preventing cross-linkages between the peptidoglycan polymer chains.

Peptidoglycan is synthesized in staphylococci by transpeptidases. A group of transpeptidases, penicillin-binding proteins (PBPs), is responsible for cleavage of cell-wall peptides at the amide bond of D-Ala-D-Ala (Ilangovan *et al.*, 2001). *S. aureus* normally produces four PBPs, which are inactivated by methicillin. The methicillin resistance is caused by PBP2a protein, which is able to continue cell wall synthesis in the presence of methicillin (Fuda *et al.*, 2004). PBP2a is not native in *S. aureus*, it is encoded by the *mecA* gene that is carried on staphylococcal cassette chromosome *mec* (SCC*mec*). Expression of methicillin resistance is heterogenous. Expression of the *mecA* is regulated with genes located on SCC*mec* as *mecR1* activator, *mecI* repressor and *mecR2* anti-repressor (Arede *et al.*, 2012). Several genetic

factors outside of the SCC*mec* have also been described affecting the methicillin resistance. Over 20 genes with association to expression of methicillin resistance were identified by transposonal mutagenesis. These genes are either factors essential for methicillin resistance (*fem*) or auxiliary (*aux*) factors (Berger-Bachi and Rohrer, 2002). The greatest impact has the *femAB* operon (Stranden *et al.*, 1997).

Methicillin has been replaced in therapy by other semi-synthetic penicillins such as flucloxacillin and dicloxacillin (Fluit and Schmitz, 2003). In comparison to other β-lactamase-resistant penicillins, it is less active, can be administered only parenterally, and has a higher frequency of rare side effects (Rivera and Boucher, 2011). Even though methicillin is no longer used as a therapeutic agent, methicillin resistance is an important indicator of resistance to other antibiotics. Methicillin-resistant bacteria are resistant to other β-lactams (penicillin, cephalosporins), moreover they often carry resistance determinants to erythromycin, clindamycin, gentamycin, ciprofloxacin, and fusidic acid (Otter and French, 2011).

2.3 Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA has become a major medical problem in the recent years. In the beginning, MRSA was predominantly a nosocomial pathogen; the first HA-MRSA strain was isolated shortly after the introduction of methicillin in clinical practice in 1961 (Jevons *et al.*, 1961). This HA-MRSA clone belonged to sequence type (ST) 250 from clonal complex (CC) 8 and carried SCC*mec* I (Enright *et al.*, 2002). Several pandemic waves of HA-MRSA with SCC*mec* I, II, or III have been observed since then (DeLeo and Chambers, 2009). Afterwards, the first case of CA-MRSA infection was documented in 1993 in Western Australia (Udo *et al.*, 1993). CA-MRSA typically carries SCC*mec* IV (Okuma *et al.*, 2002). The number of CA-MRSA infections was increasing in the 1990s and some of the CA-MRSA clones replaced typical HA-MRSA strains in hospitals. Nowadays, five main multilocus sequence type (MLST) defined pandemic clones (Table 1) ST5 (CC5), ST8 (CC8), ST22 (CC22), ST36 (CC30), and ST45 (CC45) have been established as a cause of nosocomial infections (DeLeo *et al.*, 2010). Moreover, in the past two decades there has been an increase in MRSA infections and carriage in individuals with no connection to health care, but with exposure to livestock. These LA-MRSA belong to CC398 and were first recognized among pig farmers in France and the Netherlands in the early 2000s (Armand-Lefevre *et al.*, 2005; Voss *et al.*, 2005). Since then, *S. aureus* isolates from CC398 have been reported in diverse livestock hosts in many countries around the world (Verkade and Kluytmans, 2014).

Clonal complex	Sequence type	SCC*mec* type	Common names	Geographic spread
CC5	ST5	II	USA100, New York or Japan clone	Japan, USA, UK, Europe, China, Korea
	ST5	I	EMRSA-3	Europe, UK, Japan
	ST5	IV	USA800 or paediatric clone	
	ST5	VI	HDE288 or paediatric clone (in Portugal)	Australia, Europe, UK, USA
	ST228	I	Southern German	Australia, Europe
CC8	ST250	I	Archaic	Australia, UK, Europe, Canada, USA, China
	ST247	I	Iberian clone or EMRSA-5	Australia, UK, Europe, USA
	ST239	III	Brazilian or Hungarian clone	Australia, UK, Europe, USA, Canada, China, Korea
	ST239	III	EMRSA-1	Australia
	ST8	III	AUS-2 and AUS-3	Australia
	ST8	II	Irish-1	Ireland, UK, Austria, Canada, USA
	ST8	IV	USA500, EMRSA-2 or EMRSA-6	Australia, Europe, UK, USA, Canada, China, Japan
CC22	ST22	IV	EMRSA-15	Australia, Europe, UK, New Zealand
CC30	ST36	II	USA200 or EMRSA-16	Australia, Europe, UK, Canada
CC45	ST45	II	USA600	Europe, Canada, USA
	ST45	IV	Berlin clone	Australia, UK, Europe, USA, China

Table 1: Lineages of common nosocomial MRSA strains, adapted from (Chambers and Deleo, 2009; Moore *et al.*, 2010; Robinson and Enright, 2003)

2.4 Horizontal gene transfer (HGT)

HGT is considered to have the greatest impact on the evolution of the genus *Staphylococcus.* HGT is responsible for rapid spread of various genes that were either changed due to mutation or that were acquired from different species. Computational analyses of sequenced *Staphylococcus* genomes emphasize the significance of HGT in the spread of resistance and virulence determinants carried on MGE (Suzuki *et al.*, 2012). Comparative genomics also

suggests that not only MGE but also core genes such as housekeeping genes are horizontally transferred though with lower frequencies (Chan *et al.*, 2011).

HGT is influenced by the genetic background of the recipient bacterium (Enright *et al.*, 2002). Transfer of DNA is more successful between strains from the same clonal complex or lineage (Varga *et al.*, 2012). Nonetheless, HGT extends beyond clonal complexes (Chan *et al.*, 2011), even beyond species barrier (de Niederhausern *et al.*, 2011). A comparative analysis of 324 *S. aureus* and *S. epidermidis* genomes revealed that around half of the genome was shared between the species; primarily MGEs were common to both species, suggesting their intensive HGT (Meric *et al.*, 2015).

The main barrier in DNA exchange is the restriction-modification (RM) system. Currently, there are four known RM systems, but only three types (I, II, and IV) were identified in *S. aureus* (Stobberingh *et al.*, 1977; Vasu and Nagaraja, 2013; Waldron and Lindsay, 2006; Xu *et al.*, 2011).

Type I RM system in *S. aureus* consists of one copy of *hsdR* (restriction) and two different copies of *hsdMS* (modification and specifity) genes. The *S. aureus Sau*1 system is present on chromosome or genomic islands (Holden *et al.*, 2004) nearly in all isolates of *S. aureus* (Waldron and Lindsay, 2006). On the contrary, type II RM system was found in only small number of *S. aureus* strains. Type II RM system requires genes for DNA methylases and restriction endonuclease enzymes, in *S. aureus* the *Sau*3AI endonuclease or *Sau*961 methyltransferase and endonuclease (Stobberingh *et al.*, 1977; Szilak *et al.*, 1990). The *Sau*3AI was also identified in *Staphylococcus carnosus* (Seeber *et al.*, 1990). Lastly, type IV RM system called *Sau*US1, is widespread in sequenced genomes of *S. aureus*. *Sau*US1 contains only one gene responsible for restriction of modified DNA, such as *dcm* modified cysteines from *Escherichia coli* (Xu *et al.*, 2011). Besides, RM systems were found on phage chromosomes (Dempsey *et al.*, 2005). The impact of RM systems on HGT is undoubtedly vast, yet unfortunately information about RM system in CoNS species is still scarce.

HGT comprises three distinct mechanisms: transformation, conjugation and transduction (Thomas and Nielsen, 2005). Transduction is likely to be the major method for transfer of DNA in staphylococcal populations as phages required for the transfer are prevalent among staphylococci and the process is very efficient in the laboratory (Lindsay, 2014).

2.4.1 Transformation

Transformation results from direct uptake and incorporation of exogenous DNA. It occurs naturally in some species, e.g. *Bacillus subtillis* or *Streptococcus pneumoniae* (Dubnau, 1999). For transformation to occur, these species must get into a state of competence during their life cycle, whereas other bacteria such as *Haemophillus influenzae* or *Neisseria gonorhoeae* uptake DNA quite readily anytime during their life cycle. Recently, Morikawa and collegues (2012) demonstrated that *S. aureus* can undergo natural transformation facilitated by bacterially encoded competence proteins. Expression of competence proteins is controlled by sigma H factor that is either present in high levels or have changes in its promoter. These variations occur at low frequencies; hence natural staphylococcal transformation is very rare. Transformation of staphylococci in laboratory requires preparation of protoplasts (Grüter and Laufs, 1991) or electroporation (Augustin and Gotz, 1990).

2.4.2 Conjugation

Conjugation was discovered in 1946 by Lederberg and Tatum. It is a transfer of genetic material from donor to recipient cell by a specialized multiprotein complex, termed the conjugation apparatus (Grohmann *et al.*, 2003). The donor cell must have a conjugative genetic element that is most often a plasmid or a transposon (Ryan and Ray, 2004). The conjugative plasmids encode genes *tra* that are responsible for close contact of donor and recipient cell and transport of the DNA between the two cells. The DNA transport starts with the single-stranded nick of the plasmid DNA at the *oriT*. Plasmid-encoded helicase unwinds one strand, which is transported from 5' to 3' end to the recipient cell (Llosa *et al.*, 2002).

Conjugative plasmids in staphylococcal species are most commonly 40–60 kb large plasmids. Conjugative plasmids carry often resistance determinants, such as mupirocin resistance genes on plasmid pG0400 (Morton *et al.*, 1995), gentamicin resistance on plasmid pAM899-1 (Forbes and Schaberg, 1983) and pGO1 (Projan and Archer, 1989), or multiple resistance determinants on plasmid pSK41 (Liu *et al.*, 2013). The vancomycin resistance genes were also introduced into *S. aureus* from *Enterococcus faecalis* NCTC 12201 on conjugative plasmid (Noble *et al.*, 1992).

2.4.3 Transduction

Transduction is considered to be the most effective mechanism of HGT in staphylococci. During transduction the DNA is transferred from donor into recipient bacterium by a bacteriophage. Transduction occurs when bacteriophage packages bacterial DNA instead of

viral DNA into its head and later injects this DNA into recipient cell. The length of transferred DNA is often limited by the length of the phage genome, for example transducing phages ϕ11α and ϕ80α have genomes of 43.6 and 43.9 kb, respectively (Christie *et al.*, 2010; Iandolo *et al.*, 2002). Both phages belong to serological group B, other transducing phages are from serological group F (Dowell and Rosenblum, 1962). The ability to package bacterial genes was shown also for phages from serological group A (Mašlaňová *et al.*, 2013), but their transducing potential needs to be evidenced.

There are two possible variations of transduction process – specialized and generalised transduction. During specialized transduction only certain host locus is transduced. Specialized transduction is well described for the staphylococcal pathogenicity islands (SaPIs) (Novick *et al.*, 2010). The SaPI1 is excised from chromosome during the helper-phage infection, circularized and packaged into phage particles (Tallent *et al.*, 2007). Transducing particles are smaller than the helper phage ϕ80α particles. Transducing particles transfer the SaPI1 into new recipient cells where the SaPI1 is integrated into the chromosome with high efficiency.

On the contrary, during generalized transduction the newly forming phage particles package almost any part of bacterial chromosomal or plasmid DNA (Lindsay, 2014). These pseudo-phage particles are released during the cell lysis; they can later bind to a new host, and inject the DNA into the cell, where it is either maintained or digested. If the DNA is a plasmid, it may recircularize and become plasmid again. If the DNA is a part of the bacterial chromosome, it may become recombined in the recipient chromosome. The generalized transduction of penicillinase and tetracycline plasmids was proved within *S. aureus* USA300 clones by phages ϕ80α and ϕJB (Varga *et al.*, 2012), the transduction of chromosomal locus SCC*mec* was done also in USA300 lineages by phages ϕ80α and ϕ29 (Scharn *et al.*, 2013). Many steps in the generalized transduction remain still unclear, for instance the reason why some phages are transducing and others not still remains unresolved.

2.5 Mobile genetic elements (MGEs)

MGEs are parts of bacterial DNA that are exchanged among bacteria via HGT (Malachowa and DeLeo, 2010). MGEs can have several effects on bacterial cells. Most importantly, they can carry resistance and virulence genes enhancing the pathogenicity of the bacteria. MGEs may also contribute to genome plasticity through homologous recombination or interactions of related genomic elements mediating rearrangements in chromosomal and plasmid DNA.

The main staphylococcal MGE types are bacteriophages, plasmids, staphylococcal pathogenicity islands, SCC, genomic islands, transposons, and insertion sequences.

2.5.1 Bacteriophages

The diversity of the staphylococcal genomes is determined mainly by their viruses, the bacteriophages. Phages act either as transducing vectors as described above or they reside in the genome as prophages. All known bacteriophages of staphylococci belong to the order *Caudovirales*, with three families – *Siphoviridae*, *Myoviridae*, and *Podoviridae* – characterized by tail morphology and contractility (Ackermann, 2011). Most described phages of *S. aureus* as well as of CoNS belong to the *Siphoviridae* family with a long non-contractile tail (Tkadlecek *et al.*, 1978; Xia and Wolz, 2014). Members of *Siphoviridae* family are temperate phages, they have the ability to lyse the host bacterial cells, but often they integrate in the chromosome in the form of prophage. Lytic phages of *Myoviridae* family with a long contractile double sheathed tail have the potential to be used in the phage therapy, because they lyse bacteria completely during the release of the progeny. Species of *Podoviridae* family have a very short tail and they seem to be unable to lysogenise host like the *Myoviridae* phages.

The more detailed description of bacteriophages was enabled by the improvement of molecular biology techniques, especially by genome sequencing. Nowadays, 109 complete staphylococcus phage genomes were deposited in the public Genome database, with the majority (around 86 %) of *S. aureus* phage genomes (Genome, National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/genome/, cited: 15/4/2016). Sequences of prophages from bacterial genome annotations are also available in public databases, but the prophages were usually not analysed in detail. Kwan and colleagues (2005) compared complete genomes of 27 phages and divided phages into three groups that correspond with the morphological classification: siphoviruses with 39–43 kb, myoviruses with 120–140 kb, and podoviruses with 16–18 kb genome size. The genome of siphoviruses has a mosaic structure and is usually organized into six modules: lysogeny, DNA replication, transcription regulation, packaging, head, tail, and lysis (Iandolo *et al.*, 2002). The high diversity of the phage genomes is a result of recombination and HGT of individual modules (Kahankova *et al.*, 2010), as implicated by the nucleotide sequence analysis of the genomes of phages from different serogroups.

Phages can influence the expression of virulence determinants by positive or negative lysogenic conversion. Negative lysogenic conversion occurs when function of a gene (e.g.

β-haemolyzin, *hlb*, or lipase, *geh*) is inactivated with the insertion of the phage in the bacterial genome (Coleman *et al.*, 1989; Goerke *et al.*, 2009). In positive lysogenic conversion prophages provide bacteria with additional virulence genes such as Panton-Valentine leukocidin (*lukSF*), staphylokinase (*sak*), staphylococcal inhibitor of complement (*scn*), enterotoxin A (*sea*), exfoliative toxin A (*eta*), or recently described cell-wall anchored protein SasX (Li *et al.*, 2012; Malachowa and DeLeo, 2010). Lysogeny enables bacteria to establish as a pathogen and thus has impact on the short-term evolution of bacteria.

2.5.2 Plasmids

Plasmids are DNA molecules that can exist in a cell independently of chromosome in circular or linear form and that have the ability to self-replicate. Inside the bacterium, plasmids can vary in a copy number, large plasmids have usually a low copy number while the small ones are maintained in many copies (Lanza *et al.*, 2015). These elements can be transferred via transduction, conjugation or they can be mobilised by conjugative elements (Phillips and Funnell, 2004). Plasmids can substantially affect the phenotype of their host organism, because they can carry resistance genes to antimicrobials antibiotics, heavy metals, and biocides), genes for colonization and other virulence factors or genes for other beneficial traits (bacteriocins, metabolic traits).

Several classification systems of plasmids were proposed to alleviate the study of plasmid biology. These elements were initially divided into four classes designated by Roman numerals on the basis of size, replication mechanism, ability to be transferred, phenotypic and functional characteristics, and host range (Novick, 1989; Shearer *et al.*, 2011). Class I comprises small (1.3–4.6 kb) high copy number plasmids with a rolling circle replication mechanism that typically carry one or two antibiotic resistance determinants. Class II encompasses bigger (15–45 kb) low copy number plasmids with theta replication mechanism that often harbour resistance to antibiotics, heavy metals and antiseptics. Class III includes big (30–60 kb) plasmids similar to class II that in addition carry *tra* genes responsible for conjugative transfer. In class IV, there are plasmids with distinctive features compared to the previous three classes. In the end of 1980s, Novick categorized staphylococcal plasmids into 15 incompatibility (Inc) groups based on the finding that two plasmids with the same replication control mechanism cannot stably co-exist in the same cell (Novick, 1987). Plasmids of most Inc groups correspond to class I and class II (Novick, 1989). Recently, Jensen and colleagues presented multiplex-PCR typing system based on the diversity of replication initiator proteins (Jensen *et al.*, 2010; Lozano *et al.*, 2012) and Francia with

collective suggested categorization of plasmids according to their mobilization systems (Francia *et al.*, 2004; Garcillan-Barcia *et al.*, 2009). Nowadays, the whole genome sequencing provides the most comprehensive information on plasmids.

Substantial number of plasmids found in staphylococci carry resistance genes to antibiotics. Class I plasmids encode often resistance to tetracycline, chloramphenicol, macrolides, and trimethoprim. Class II plasmids are often responsible for resistance to β-lactams, aminoglycosides, and macrolides, heavy metals such as arsenic, cadmium, and mercury, and/or antiseptics (quaternary ammonium compounds) (Malachowa and DeLeo, 2010). Resistance genes are also localized on transposons that integrate in plasmid DNA and can cause rearrangement of these elements (Thomas and Archer, 1989). Virulence genes, for instance enterotoxin D and J (Zhang *et al.*, 1998) and exfoliative toxin B (Yamaguchi *et al.*, 2001), were also found on plasmids. Considering ubiquitous distribution of these extrachromosomal elements, plasmids undeniably play substantial role in the evolution of staphylococcal lineages as 89 % of MRSA strains in Czech hospitals were found to have one or more plasmids in a study by Kuntová *et al.* (2012).

2.5.3 Staphylococcal cassette chromosome (SCC)

SCC is a 21–67 kb long mobile genetic element (Kuroda *et al.*, 2001) that inserts itself at 3' end of the *orf*X gene (also termed *rlm*H) encoding a ribosomal methyltransferase (Boundy *et al.*, 2013). The SCC has a mosaic structure and can carry multiple genes for resistance factors. One of the resistance determinants carried within this MGE may be the *mecA* gene responsible for methicillin resistance in staphylococci. SCC with *mecA* gene are designated SCC*mec*. To date, 12 types of SCC*mec* elements (I – XII) have been described (IWG-SCC, 2009; Wu *et al.*, 2015). The SCC*mec* classification is based on the combination of two gene complexes: the *mec* gene complex, containing the *mecA* gene, and the chromosome cassette recombinase (*ccr*) gene complex, containing one *ccr*C or two *ccr*AB serine recombinases (Shore and Coleman, 2013). The SCC*mec* types are described in Table 2 and compared in Figure 2.

SCC*mec* structure is usually very complex; apart from *mecA* it can carry other resistance determinants. Plasmid pUB110 in SCC*mec* II encodes resistance to aminoglycosides, Tn*554* in SCC*mec* II and III encodes resistance to erythromycin, plasmid pT181 in SCC*mec* III encodes resistance to tetracycline and mercury, and pseudo Tn*554* in SCC*mec* III encodes resistance to cadmium (Ito *et al.*, 2001; Ito *et al.*, 1999). Furthermore, the virulence genes such as *pls* and *pms* were also found located on SCC*mec* (Queck *et al.*, 2009; Savolainen *et*

al., 2001). Therefore acquisition of SCC*mec* by pathogenic staphylococci can raise severe medical problems. Above all, the MRSA strains that obtained SCC*mec* through HGT cause difficulties in treatment of staphylococcal infections.

Feature	SCC*mec* Allotype											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Size (kb)	34	53	67	21	24	28	24	32	32	51	29	25
***mec* gene complex class**	B	A	A	B	C2	B	C1	A	C2	C1	E (*mecC*)	C2-like
***ccr* genes**	A1B1	A2B2	A3B3	A2B2	C1	A4B4	C1	A4B4	A1B1	A1B6	A1B3	C2
***ccr* gene complex type**	1	2	3	2	5	4	5	4	1	7	8	9
Other resistance genes	none	*ermA*	*ermA*, *tet(K)*, *mer*	none	none	none	none	*ermA*, *spc*	*cadDX*, *arsRBC*, *arsDARBC*	*cadDX*, *arsRBC*	*arsRBC*, *blaZ*	none
Representative *S. aureus* strain	COL	N315	85/2082	MW2	PM1	HDE288	JCSC6082	BK20781	JCSC6943	JCSC6945	LGA251	BA01611

Table 2: SCC*mec* allotypes. Abbreviations: kb – kilobases, *ermA* – erythromycin resistance gene, *tet* – tetracycline resistance gene, *mer* – mercury resistance gene, *spc* – streptomycin resistance gene, *cadDX* – cadmium resistance genes, *arsRBC*, *arsDARBC* – arsenic resistance genes, *blaZ* – β-lactamase

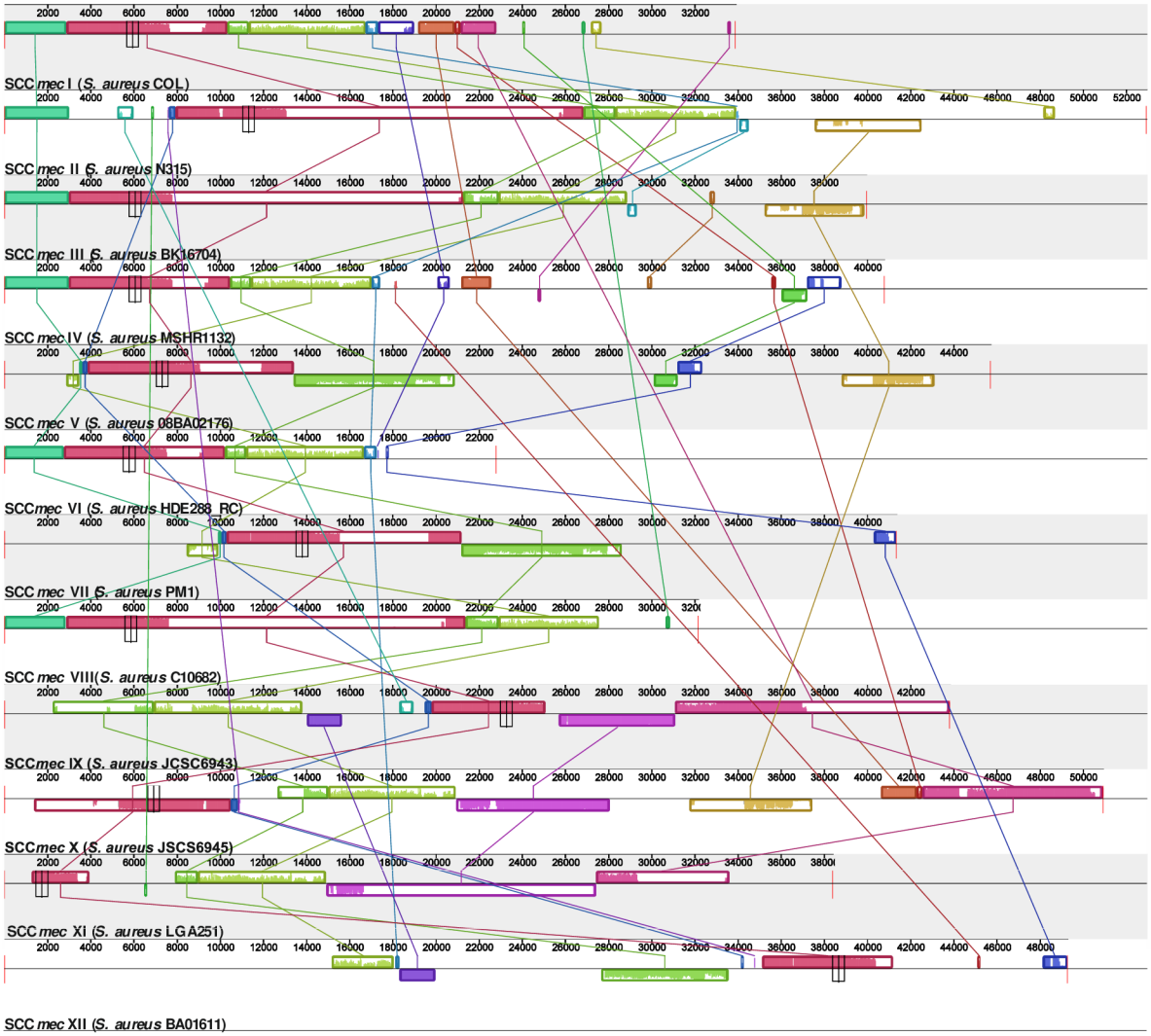

Figure 2: Comparison of 12 SCC*mec* types using Mauve v.2.3.1.

SCC*mec* types I, II, and II are considered to be the evolutionary oldest SCC*mec* elements in *S. aureus.* SCC*mec* type I was found in *S. aureus* in the 1960s and 1970s right after the introduction of methicillin (Ito *et al.*, 2001). These isolates are known as archaic clones. SCC*mec* type II was first described in the strain N315 commonly known as New York/Japan (Ito *et al.*, 1999). The SCC*mec* type III was first found in HA-MRSA strain 85/2082 (Ito *et al.*, 2001). It is considered to be the largest SCC*mec* type with 67 kb but is actually comprised of a SCC*mec* of 35 kb and an adjacent SCC element, SCC*mercury* (SCC*mer*) of 32 kb. SCC*mec* elements I, II and III were often found in isolates from hospital environment, probably because they contain additional resistance factors, such as pUB110 and pT181 plasmids, and/or Tn554.

SCC*mec* type IV is shorter than SCC*mec* I, II or III, with 21-31 kb on average (Ma *et al.*, 2002). It was first described in *S. aureus* MW2 and is most commonly found in CA-MRSA, however it has also been described in the epidemic HA-MRSA, e.g. EMRSA-15 (Ito *et al.*, 2003; Ma *et al.*, 2002).

SCC*mec* type V is another commonly found SCC*mec* type in CA-MRSA. The first SCC*mec* V element of the size of 28.6 kb was detected in an Australian strain WIS (Ito *et al.*, 2004). In contrast to previously described SCC*mec* types that encode CcrA and CcrB recombinases it carries gene for only one recombinase, CcrC.

SCC*mec* type VI, VII, VIII, IX, X, XI, and XII are detected only sporadically. SCC*mec* VI was first described in a MRSA from a paediatric hospital in Portugal in 1992 which became known as the “paediatric clone” (Oliveira *et al.*, 2006). SCC*mec* type VII was first found in strain JCSC6082 from Sweden. Its J3 region has 99% homology to regions SCC*mec* III strain 85/2082 (Berglund *et al.*, 2008). SCC*mec* type VIII was first detected in 2003 in Canada in MRSA C10682 (Zhang *et al.*, 2009). SCC*mec* type IX was first described in 2011 in strain JCSC6943 by Li and colleagues (2011). The same collective also discovered SCC*mec* type X in strain JCSC6945. Recently described SCC*mec* type XI from *S. aureus* strain LGA251 carries a homologue to *mecA* gene designated as the *mecC* (Becker *et al.*, 2014; Garcia-Alvarez *et al.*, 2011). The *mecC* shares 70% nucleotide identity with *mecA* and was identified in *S. aureus* from numerous sources including humans and a range of animals (Cuny *et al.*, 2011; Garcia-Alvarez *et al.*, 2011), as well as in *Staphylococcus xylosus* (Harrison *et al.*, 2013) and *S. sciuri* (Harrison *et al.*, 2014). The *mec* gene complex from *S. aureus* LGA251 with *mecC* gene is structurally similar to *mec* gene complex from *Macrococcus caseolyticus* with *mecB* gene that was found in the plasmid pMCCL2 (Baba *et al.*, 2009) and SCC*mec*-like

element in the *M. caseolyticus* chromosome (Tsubakishita *et al.*, 2010a). The latest described SCC*mec* type XII was identified in *S. aureus* strain BA01611 isolated from a milk sample of a Holstein cow with clinical mastitis (Wu *et al.*, 2015). It contains novel *ccrC2* gene encoding for CcrC2 recombinase, which is able to excise the SCC element from the chromosome as CcrAB and CcrC recombinases, enabling the spread of these elements.

SCC elements are not found across the whole genus *Staphylococcus*. The origin of SCC*mec* is still discussed, but it was suggested that *S. aureus* acquired the element from CoNS. This mobile element could originate in *Staphylococcus sciuri* group and it seems that *S. fleurettii* was the source of the *mecA* region (Tsubakishita *et al.*, 2010b). Many SCC*mec* and related elements were found in CoNS such as *S. epidermidis* (Wisplinghoff *et al.*, 2003), *S. hominis* (Bouchami *et al.*, 2011), *S. haemolyticus* (Urushibara *et al.*, 2011), and other species (Perreten *et al.*, 2013; Vanderhaeghen *et al.*, 2012; Zong *et al.*, 2011).

Objectives of the diploma thesis

The main focus of this thesis is to study the role of horizontal gene transfer in the evolution of genus *Staphylococcus*. This includes examination of the role of phages in the horizontal gene transfer among selected species of the genus *Staphylococcus* and characterization of transferable elements with a focus on evolution of SCC, which have particularly significant impact on dissemination of feared MRSA clones.

Overall, the particular objectives are:

1. Design and implementation of a new method for rapid screening of reactivable prophages.
2. Isolation of new inducible CoNS phages and characterization of lysogenic and susceptible strains.
3. Assessment of transduction potential of the new CoNS phages:
    a. preparation of bacterial strains suitable for the transduction experiment assays, including optimization of the electroporation method,
    b. optimization of transduction in broth-based approach.
4. Characterization of selected MGEs of CoNS.
5. Phylogenetic analysis of the SCC elements.

3 Material and methods

3.1 Bacterial strains and bacteriophages

Bacterial strains of *Staphylococcus sciuri*, *Staphylococcus lentus*, and *Staphylococcus petrasii* were clinical isolates (National Reference Laboratory for Staphylococci (NRL/St), The National Institute of Public Health, Praha, Czech Republic) and they were obtained from Czech Collection of Microorganisms (CCM) under designation listed in the Table 3.

Species	Subspecies	CCM designation	Species	Subspecies	CCM designation
S. petrasii	*jettensis*	CCM 8494	*S. petrasii*	*jettensis*	P5415
	petrasii	P3749		*pragensis*	P5416
	petrasii	P3750		*petrasii*	P5562
	petrasii	P3751		*petrasii*	P5563
	petrasii	P3752		*petrasii*	P5564
	petrasii	P3753		*petrasii*	P5565
	petrasii	P4317		*pragensis*	P5566
	petrasii	P4318		*petrasii*	P5643
	petrasii	P4320		*pragensis*	P6089
	croceilyticus	P4322		*petrasii*	P6090
	croceilyticus	P4323		*pragensis*	P6102
	croceilyticus	P4325		*petrasii*	P6103
	croceilyticus	P4326		*jettensis*	P6317
	pragensis	P5396		*jettensis*	P6318
	pragensis	P5397	*S. sciuri*		P543
	pragensis	P5398			P548
	croceilyticus	P5400			P553
	jettensis	P5401			P569
	jettensis	P5402		*carnaticus*	P575
	pragensis	P5403		*carnaticus*	P581
	jettensis	P5404			P589
	petrasii	P5405			P590
	petrasii	P5406			P602
	petrasii	P5407			P607
	petrasii	P5408			P611
	petrasii	P5409		*rodentium*	P612
	jettensis	P5410		N.D.	P723
	jettensis	P5411		N.D.	P879
	jettensis	P5413	*S. lentus*		P583

Table 3: Bacterial strains of CoNS used in this work. N.D. – not defined

The restriction deficient bacterial strain *Staphylococcus aureus* RN4220 was used as a recipient for the transduction experiments. *S. aureus* strain NRL/St 07/759 was used as a donor of a penicillinase plasmid with cadmium resistance. *S. aureus* strains COL and Jevons B were also used in this work. The control DNA of *Bifidobacterium breve* ATCC 15700 was used in transduction assays.

Phages ϕ575, ϕ581, and ϕ879 used in this work were induced from *S. sciuri* strains P575, P581, and P879 respectively, phage ϕ5404 was reactivated from *S. petrasii* strain P5404.

3.2 Growth of bacterial strains and phages

The bacterial cultures were grown either in the meat peptone broth (MPB) made from 13 g nutrient broth CM001 (Oxoid, Basingstoke, UK), 3 g yeast extract LP21 (Oxoid), and 5 g peptone L37 (Oxoid) dissolved in 1 000 ml distilled water (pH 7.4); or in the 2× yaest trypton (2YT) medium made from 16 g tryptone LP42 (Oxoid), 10 g yeast extract LP21 (Oxoid), and 5 g NaCl (Penta, Prague, Czech republic) dissolved in 1 000 ml distilled water (pH 7.4). The stock solutions of bacterial cultures were kept frozen at -70°C or -20°C in the Hogness modified freezing medium (HMFM) (3.6 mM $K_2HPO_4 \times 3H_2O$; 1.3 mM KH_2PO_4; 2 mM natrium citrate; 1 mM $MgSO_4 \times 7H_2O$; and 4.4% (v/v) glycerol). Routine bacterial growth was done on meat peptone agar (MPA) plates with 1.5% nutrient agar, pH 7.4 (nutrient agar CM3, Oxoid) overnight (18 h) at 37°C. The brain-heart infusion (BHI) agar (47 g brain heart infusion CM1136 (Oxoid) dissolved in 1 000 ml distilled water, pH 7.3), tryptone soya agar (TSA) (40 g tryptone soya agar CM131 (Oxoid) dissolved in 1 000 ml distilled water, pH 7.3), or 2YT agar 16 g tryptone LP42 (Oxoid), 10 g yeast extract LP21 (Oxoid), 5 g NaCl (Penta), and 15 g agar No. 3 LP13 (Oxoid) dissolved in 1 000 ml distilled water, pH 7.4) were also used, especially for the phage multiplication.

The phage propagation was performed on previously described agar plates poured with 2.5 ml of 0.7% soft agar (7 g agar No.1 L11 (Oxoid), 13 g nutrient broth CM1 (Oxoid), 5 g peptone L37 (Oxoid), and 3 g yeast extract LP21 (Oxoid) dissolved in 1 000 ml distilled water, pH 7.4) with 100 µl of the overnight culture with addition of $CaCl_2$ (Penta) to final concentration 2 mM, and with the 100–400 µl of the phage lysate. The plates were incubated overnight at 37°C. After the incubation the plates were poured with 3 ml of MPB, the soft agar was resuspended with the microbiological hockey stick; the fluid was transferred to falcon tubes and centrifuged twice for 15 min at 3 100 rpm at 10°C. The supernatant was passed through the 0.45 µm filter (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and stored at 4°C. Alternatively, phages were propagated in the MPB. 20 ml MPB with 0.8 ml of the

overnight bacterial culture was incubated in the aerator for 1.5 h at 37°C. Then 3 ml of phage lysate and $CaCl_2$ to final concentration 2 mM were added into the aerator and incubated for 1.5 h at 37°C. After the incubation the aerators were left in the fridge (4°C) overnight. The next day the lysate was centrifuged 15 min at 4 500 rpm at 10°C, passed through the 0.45 µm filter, and stored at 4°C. The number of plaque forming units in the phage lysate was determined using the soft agar spot assay. The sterility of the lysate was examined by spreading 250 µl of the lysate on the MPA.

3.3 UV-reactivation of prophages

10 ml of the overnight culture was transferred into the 50 ml MPB in the Erlenmeyer flask and incubated 2 h at 37°C with shaking. 45 ml of the grown bacterial culture was transferred in the sterile Falcon tube and centrifuged (15 min, 3 000 rpm, 8°C). The pellet was resuspended in the physiological solution (8.5 g NaCl (Penta) dissolved in 1 000 ml of distilled water) and diluted to $OD_{600} = 0.15$. 10 ml of the diluted culture was poured in sterile Petri dish and UV-irradiated for 20–25 s using a 15 W lamp in 50 cm distance. The irradiated suspension was transferred into the sterile aerator together with 30 ml of physiological solution and 10 ml of 10× concentrated prophage broth (13 g nutrient broth CM1 (Oxoid), 2 g NaCl (Penta), 2 g yeast extract LP21 (Oxoid), and 10 g tryptone LP42 (Oxoid) dissolved in 100 ml of distilled water). The aerators were incubated 2 h at 37°C with moderate aeration and then kept in the fridge (4°C) until next day. The lysates were centrifuged (10 min, 5 000 rpm, 4°C), passed through the 0.45 µm filter, and the number of plaque forming units in the phage lysate was determined using the soft agar spot assay.

3.4 UV-reactivation of prophages on 96-well plate

20 µl of the overnight culture was added in a well of culture plate with 180 µl MPB. The plates were incubated 2 hours at 37°C with shaking (120 rpm). After the centrifugation (15 min, 3 000 rpm) the pellet was resuspended in 200 µl of physiological solution. The plates without the lid, rapidly shaking, were UV-irradiated for 25 s or 35 s using a 15 W lamp in 50 cm distance. 40 µl of the irradiated suspension was transferred in a new plate together with 120 µl of physiological solution and 40 µl of 10× concentrated prophage broth per well. The plates were protected from light and incubated at 37°C for 2 hours. The plates were then centrifuged (10 min, 4 000 rpm) and the supernatant was passed through the 0.45 µm filter into a sterile microtube.

3.5 Reactivation of prophages with mitomycin C

Phages were also reactivated chemically with mitomycin C according to the protocol adapted from Pulverer *et al.* (1974). 5 µl of mitomycin C (2 mg/ml) was added to 10 ml of the overnight culture. The culture was incubated 2 h at 37°C with shaking and another 2 h at 37°C without shaking. The culture was centrifuged (20 min, 3 000 rpm), 5 ml of the supernatant was transferred to a sterile tube, and the sediment was resuspended in 5 ml MPB. The supernatant and the resuspended sediment were incubated 2 h at 37°C and centrifuged 5 min at 5 000 rpm Afterwards both supernatants were passed through 0.45 µm filter and stored at 4°C.

3.6 Phage propagation from one plaque

Isolated plaques of the phages were prepared by addition of 100 µl of UV- or chemically reactivated phage lysate in the appropriate dilution into the 0.7% soft agar with 100 µl of the overnight culture with addition of $CaCl_2$ in final concentration 2 mM followed by pouring on the agar plates and overnight incubation. One isolated plaque was taken to 100 µl of MPB, resuspended and passed through small bacteriological filter (0.45 µm) into 500 µl of susceptible bacterial culture (OD_{600} = 0.2) with addition of $CaCl_2$ in final concentration 2 mM. The suspension was incubated for 2 h at 37°C rapidly shaking (900 rpm). Then the lysate was centrifuged at 13 000 rpm for 15 min and passed through 0.45 µm filter. The acquired lysate was used for further propagation of the phages on the plates as described in section 3.2.

3.7 Phage adsorption rate assay

5 ml of the overnight culture with $CaCl_2$ (c = 2 mM) was mixed with 0.5 ml of the phage lysate and incubated at 37°C in water bath, shaking. 500 µl of this suspension was removed in time 0, 5, 10, 15, 20, and 30 min and centrifuged at 10 000 rpm for 3 min in desktop microcentrifuge Mini spin plusR Eppendorf. The number of plaque forming units (PFU) was detected in the starting lysate and in the supernatants on propagation strain by soft agar assay. Briefly, 100 µl of the overnight culture with addition of $CaCl_2$ in final concentration 2 mM was added into a tube with 2.5 ml of the 0.7% soft agar. The serial dilution of the phage lysate was prepared in the MPB to 10^{-3} and 100 µl of each dilution was added into the tube. The MPA plates were poured over with the soft agar, left to dry, and incubated at 37°C overnight. The plaques were counted at appropriate dilution and the PFU was calculated.

3.8 Purification of phage particles in CsCl

Firstly, 320 ml of phage lysate was centrifuged at 23 000 rpm for 2.5 h. The pellet was dissolved in 200 µl of phage buffer (10 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl with pH). Secondly, phages were centrifuged at 40 000 rpm for 3.5 h in step gradient of CsCl. The CsCl step gradients were determined by the density of the CsCl solutions (1.45; 1.5; and 1.7 g/ml) prepared in SM buffer (0.58 g NaCl, 0.2 g $MgSO_4 \times 7\ H_2O$, and 5 ml of 1M Tris-HCl buffer with pH 7.5 dissolved in 100 ml of distilled water). The phage particles were purified from the CsCl in the dialysis tubing (VISKING 8/32, Roth) in phage buffer overnight.

3.9 Determination of inhibitory and sub-inhibitory concentration

The sub-inhibitory concentration of tetracycline was determined in liquid antibiotic medium. 100 µl of the overnight culture was added into the tube with 3 ml of antibiotic medium (17.5 g antibiotic medium No. 3 CM287 (Oxoid) dissolved in 1 000 ml, pH) with different concentrations of the inhibition agent tetracycline 0.25; 0.50; 1.00; 2.00; 4.00; 6.00; 8.00; 10.00; 12.00; and 15.00 µg/ml. The tubes were incubated overnight at 37°C. The next day the OD_{600} was measured and the sub-inhibitory concentration was determined.

The sub-inhibitory and inhibitory concentration of cadmium was determined on plates. 100 µl of the overnight culture was spread with microbiological hockey stick on MPA plates with different concentration of cadmium (1×10^{-4}; 7.5×10^{-5}; 5×10^{-5}; 2.5×10^{-5}; 1×10^{-5} M prepared from stock solution of $Cd(NO_3)_2$ with c = 10 mM) The plates were incubated overnight at 37°C. The next day the growth of the bacteria was assessed and the sub-inhibitory concentration was determined.

3.10 Electroporation

The electroporation-based transformation of staphylococci was adapted according to Lee (1995), and Kraemer and Iandolo (1990).

The competent cells were prepared according to Lee (1995) in glycerol as follows: 3 ml of B2 broth (10 g hydrolysed casein, 25 g yeast extract, 5 g glucose, 25 g NaCl, 1 g K_2HPO_4 dissolved in 900 ml of distilled water, pH adjusted to 7.5 and distilled water added to final volume 1 000 ml) was inoculated with 3 µl of stock bacterial and incubated at 37°C overnight. 1.5 ml of the overnight culture was added into the 150 ml B2 broth in 1 l Erlenmeyer flask and incubated at 37°C with shaking (250 rpm) until the OD_{600} reached 0.8–0.85. The cells were kept on ice for 15 min and transferred into the sterile 500 ml centrifugation tubes. After the centrifugation (12 000 g, 15 min, 4°C) the supernatant was decanted and the pellet was resuspended in 500 ml of cold sterile distilled water and

centrifuged again (12 000 g, 15 min, 4°C). The cells were kept on ice during the manipulation. This was repeated twice. The pellet was then resuspended in 25 ml of cold sterile 10% glycerol and transferred into the 30 ml centrifugation tubes. After the centrifugation (12 000 g, 15 min, 4°C) the supernatant was decanted and the cells were resuspended in 20 ml of 10% glycerol and incubated 15 min at 20°C. Afterwards, the tubes were centrifuged (12 000 g, 15 min, 4°C) and the pellet was resuspended in 2 ml of 10% glycerol. The final concentration of cells was expected to be 10^{10} colony forming unit (CFU). The cell suspension was divided into 250 µl aliquots and stored at -70°C.

The second approach of competent cells preparation was done according to Kraemer and Iandolo (1990) in sucrose as described: 0.5 ml of the overnight bacterial culture was added into 50 ml Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl dissolved in 1 000 ml of distilled water, pH 7.0) and incubated at 30°C until the OD_{600} reached 0.4 – 0.7. Afterwards the cultures were kept on ice for 30 min, centrifuged (10 min, 7 000 rpm, 4°C), and the supernatant was decanted. The pellet was resuspended in 40 ml of 0.5M sucrose, centrifuged (10 min, 8 000 rpm, 4°C), the supernatant was decanted, the pellet was resuspended in 20 ml of 0.5M sucrose, centrifuged (10 min, 8 000 rpm, 4°C), the supernatant was decanted, the pellet was resuspended in 10 ml of 0.5M sucrose, centrifuged (10 min, 8 000 rpm, 4°C), the supernatant was decanted, and the pellet was resuspended in 250 µl of 0,5M sucrose. The bacterial suspension was divided into 50 µl aliquots and stored at -70°C.

After the competent cell preparation the plasmid DNA was electroporated into the bacteria. 10 µl of the pT181 plasmid DNA (c = 27.8 ng/µl) or 5 µl of the pUSA300-HOUMR-like plasmid DNA (c = 45.0 ng/µl) was pipetted into a sterile microtube. Competent cells were thawed at room temperature and 50 µl of the cells was added into the microtube. The solution was incubated 30 min at room temperature. The mixture of cells and DNA was transferred into 0.2 cm electroporation cuvette (Biorad) and inserted in the Gene Pulser Xcell (BioRad). The electroporation conditions were set to C = 25 µF, PC = 100 Ω, V = 2.9 kV. For each sample, 1 ml of SMMP medium with sub-inhibitory concentration of the selection agent was added into the cuvette. The SMMP was prepared from 55 ml of 2× SMM, 40 ml of 4× Penassay broth (17,5 g of antibiotic medium No. 3 CM287 (Oxoid) in 250 ml of distilled water), and 5 ml of 10% bovine serum albumin, pH was adjusted to 7.0 and the SMMP solution was sterilised by filtration through 0.45 µm bacteriological filter; SMM was prepared from 25 ml of 0.2M sodium maleate and 40 ml of 0.1M NaOH, pH was adjusted to 6.5 and 5 ml of 1M $MgCl_2$, 42.7 g of sucrose, and distilled water to final volume 125 ml was added.

The electroporated cells were transferred into a sterile tube and incubated 1 h at 37°C with shaking (250 rpm). After the incubation the electroporated cells were spread on TSA plates with the tetracycline (c = 5 µg/ml) or cadmium (c = 5 nM) in volume of 50 µl, 100 µl, and 200 µl in triplicates and incubated at 37°C for 48 h.

3.11 Transduction

Control DNA of *Bifidobacterium breve* ATCC 15700 was added to the transducing lysate. 10 µl of Norgen's RNase-Free DNase I was added and the lysates were incubated for 15 minutes at room temperature, followed by DNase I inactivation at 75°C for 5 minutes. Then bacterial suspension was mixed with lysate and $CaCl_2$ was added into the mixture to final concentration 2 mM. The mixture was incubated 25 min at 37°C in water bath, shaking. Sodium citrate was added to the mixture to final concentration 0.015 M and the mixture was centrifuged (3 000 rpm, 10 min, 4–8°C). The pellet was resuspended in 300 µl of 0.017M sodium citrate and spread on TSA plates with the selection agent (tetracycline, c = 5 µg/ml) and sodium citrate (c = 20mM). The transductions were evaluated after 48 h incubation at 37°C, potential transductants were transferred on new TSA plates with selection agent and sodium citrate, incubated overnight (37°C), transferred on TSA plates with sodium citrate only, incubated overnight (37°C), transferred on MPA plates, incubated overnight (37°C), and transferred to HMFM for keeping in -70°C until further analyses.

3.12 DNA Isolation

The bacterial DNA was isolated using the commercially available High Pure Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with modifications of the cell lysis. Briefly, 7 ml of the overnight culture was washed twice in phosphatebuffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and resuspended in 235 µl of Suspension Buffer (Roche) + 15 µl of lysostaphin (Sigma, stock concentration 10 mg/ml). Then, it was left incubating at 37 °C to lysis (max 2 hours). After the treatment, the manufacturer's protocol was followed.

3.13 Template preparation for PCR by cell lysis

500 µl of MPB was inoculated by 5 µl of stock culture and incubated 24 h at 37°C. The suspension was then centrifuged (12 000 rpm, 3 min), supernatant was discarded and pellet was washed in 1 ml distilled water. After the centrifugation (12 000 rpm, 3 min) the pellet was resuspended in 1 ml of distilled water and placed for 20 min at 80°C in Dry Block Heater (TS100 Theromshaker, Biosan). The lysate was stored at -20°C for a few days and before the use as a template for PCR it was centrifuged for 1 min at 14 000 rpm.

3.14 Plasmid DNA isolation

Plasmid DNA was isolated using the commercially available NucleoSpin Plasmid Isolation Kit (Macherey-Nagel, Germany). The isolation was done according to manufacturer's protocol with modification of the cell lysis. 7 ml of the overnight culture was centrifuged (4 500 rpm, 15 min), supernatant was discarded and pellet was washed in 5 ml PBS. The suspension was centrifuged again (4 500 rpm, 15 min) and entire supernatant was discarded. The pellet was resuspended in lysis solution made from 235 µl solution A1 (Macherey-Nagel) and 15 µl lysostaphin. The lysis mixture was incubated at 37°C until the cell lysed. Afterwards the plasmid DNA was isolated following the manufacturer's protocol.

3.15 Restriction analyses of plasmid DNA

Plasmid DNA was digested for routine examination of the restriction pattern with *Hin*dIII (Roche Diagnostics) endonuclease according to the manufacturer instruction. Enzymes *Apa*I, *Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Not*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I (all Roche Diagnostics) were also used for the restriction pattern analyses according to the manufacturer's instruction. Restriction fragments length polymorphism (RFLP) was detected by agarose gel electrophoresis.

3.16 PCR

The isolated plasmid DNA or cell lysate were used as templates for PCR. FastStart PCR Master (Roche Diagnostics) and primers (listed in Table 4) in final concentration 200 nM were used for the preparation of PCR mix. PCR Water (Qiagen) was added to the mix up to the volume 25 µl per sample. The PCR condition were set to initial 5 min denaturation at 95°C, 30 cycles of denaturation (30 s, 94°C), annealing (55°C, 30 s), and extension (72°C, 45s). The final extension was set for 4 min at 72°C. PCR products were detected by agarose gel electrophoresis.

Detected gene	Primer	Sequence (5'–3')	Size (bp)	Reference
tetK	tetK-F	TCGATAGGAACAGCAGTA	169	(Ng *et al.*, 2001)
	tetK-R	CAGCAGATCCTACTCCTT		
cadD	cadDX-F	GGATATTAGGTTTATTGGGT	162	(Kuntová *et al.*, 2012)
	cadDX-R	CGCCACAACTTGCTATCGTA		
blaZ	blaZ-F	ACTTCAACACCTGCTGCTTTC	173	(Martineau *et al.*, 2000)
	blaZ-R	TGACCACTTTTATCAGCAACC		

Table 4: Primers used for PCR detection of resistance genes

3.17 Relative quantification analysis

The relative quantification analysis was used to provide the ratio between bacterial targets either chromosomal (part of SCC*mec*) or plasmid-borne (kanamycin nucleotidyltransferase) and phage reference (adaptor protein between head and tail). The LightCycler 490 Real-Time PCR Instrument (Roche) was used for relative quantification analysis. Reactions were carried out in triplicates in a MicroAmp® optical 96-well reaction plates sealed with optical adhesive covers (Roche). Each reaction mixture (10 µl) contained 7.5 µl FastStart® TaqMan Probe Master (Roche), 900 nM of each primer (Table 5), 250 nM of hydrolysis probe (Table 5), and 2.5 µl of template DNA. An initial denaturation of DNA at 95°C for 10 min was followed by 40 cycles of amplification (95°C for 15 s and 60°C for 45 s).

The relative quantification analysis was performed as dual colour and in run experiment. Two different dyes, FAM and Cy5, were used in conjunction with probes. The FAM probes with absorption maximum at 494 nm and emission maximum at 518 nm were used for SCC*mec* and kanamycin nucleotidyltransferase, the Cy5 probes with absorption maximum at 650 nm and emission maximum at 670 nm were used for detection of phage adaptor protein between head and tail. The results were generated by the Light-Cycler software using the second derivate maximum and one target gene was paired with one reference by one to one pairing analysis. The reaction efficiency was calculated for each analysed gene and the results were normalized according to the efficiencies in the relative quantification.

Detected region	Oligonucleotide	Sequence (5'–3')
Fragment of SCC*mec*	Primer MecFW	CATTATTCCAGGCATGA
	Primer MecRV	CTACATTGTTTCGGTCT
	Probe Mec	CGTTCTGATTTCAATGGTTCAA
Kanamycin nucleotidyltransferase	Primer KanRFW	CATCTGTGCCAGTTCGTA
	Primer KanRRV	AAATTCTCTAGCGATTCCAG
	Probe KanR	CTCAGAGTCGGAAAGTTGACC
Adaptor protein between head and tail	Primer TailFW	TAAAAGCTTATTATGAGTGGG
	Primer TailRV	TAATAGTACGCTGTTAGTGGA
	Probe Tail	ATTCATCAGTTACTGCACTCG

Table 5: Primers and probes used for qPCR designed by Zeman (2015)

3.18 Agarose gel electrophoresis

Plasmid DNA, length of the plasmid fragments after the restriction and PCR products were analysed by one step agarose gel electrophoresis. TAE (4.8 g of Trizma® base (Sigma), 1.1 ml of glacial acetic acid (Penta), and 2 ml of 0.5M EDTA (Sigma) brought to final volume

up to 1 l with distilled water) was used as electrophoretic buffer. The 1.2% agarose gel was made from agarose (SERVA Electrophoresis, Germany) and TAE buffer. Voltage used in the electrophoretic bath was 5 V/cm between electrodes. After the electrophoresis the gel was stained with ethidium bromide solution (c = 1 µg/ml) and visualised using a UV-transilluminator.

3.19 Pulse-field gel electrophoresis (PFGE)

Macrorestriction profiles of possible transformants and transductants were examined by PFGE. 100 µl of 0.5 M EDTA was added to cells in 10 ml MPB after overnight incubation at 37°C. The cells were centrifuged (4 000 rpm, 10 min, 4°C) and washed twice in wash solution (10 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 1M NaCl, pH 7.5). The harvested cells were resuspended in wash solution and the OD_{600} was adjusted to 0.2–0.35. 10 ml of this suspension was centrifuged (4 000 rpm, 10 min, 4°C) and pellet was resuspended in 100 µl of wash solution. The suspension was heated to 55°C for 2–3 min. For plug preparation 110 µl of 2% low-melting point agarose (Sigma) containing 10 µl 0.5 mg/ml lysostaphin (Sigma) was mixed with the cell suspension. Plugs were incubated in 1 ml lysis solution (6 mM Tris-HCl, 100 mM EDTA, 1M NaCl, 0.5% Brij 58, 0,2% sodium deoxycholate, 0.5% N-laurylsarcosin, pH 7.6) with 10 µl of 0.5 mg/ml lysostaphin at 37°C in a water bath at 60rpm for 3 h. The lysis solution was exchanged with 1ml of deproteinization solution (20mM EDTA, 20 mM EGTA, 1%, pH 9.0) with 25 µl of proteinase K (20 mg/ml). Plugs were incubated at 55°C in a water bath at 60 rpm for 6 h, then the deproteinization solution and proteinase K was exchanged and the incubation continued for another 6 h. Afterwards, the plugs were washed 5 times with 10 ml TE, pH 8.0 (10 mM Tris, 1 mM EDTA) and kept at 8°C.

Slices of the DNA containing agarose plugs were incubated for 4 h in the presence of 10 U *Sma*I (Roche Diagnostics) following the manufacturer's instruction. DNA restriction fragments were separated in 1.2% pulsed field certified agarose gels (Bio-Rad) at 6 V/cm in 1×TAE using a CHEFF MAPPER (Bio-Rad). The pulse times were ramped from 1 to 70 s for 24 h at 14°C and 120°. Gels were stained with ethidium bromide and visualised using aUV-transilluminator.

3.20 Phage genomic DNA isolation

Phage DNA was isolated with the Phage DNA Isolation Kit (Norgen Biotek Corporation, Canada). Firstly, DNase treatment was performed to remove any host genomic DNA. Control DNA of *Bifidobacterium breve* ATCC 15700 was added to the 1 ml of purified phages. Then

10 µl of Norgen's RNase-Free DNase I was added and the tubes were incubated for 15 minutes at room temperature, followed by DNase I inactivation at 75°C for 5 minutes. Secondly, phage particles were lysed. 500 µl of Lysis Solution was added in the tube, vortexed, and 4 µl of Proteinase K (20 mg/ml, Roche Diagnostics, Germany) was added. Tubes were incubated at 55°C for 30 minutes, followed by incubation at 65°C for 15 minutes. Thirdly, the DNA was isolated according to the manufacturer's protocol. The DNA was stored at 4°C.

3.21 DNA sequencing

DNA sequencing was performed using the Ion Torrent™ Personal Genome Machine (Ion PGM™) in conjunction with Ion Torrent™ workflow reagents (Life Technologies, UK) by a Ion Torrent sequencing platform operative similarly as described in (Stanczak-Mrozek *et al.*, 2015). Sequence assembly was performed using SPAdes v3.1.0 (Nurk *et al.*, 2013) with default parameters for Ion Torrent data.

Sequences were manipulated and inspected in cross platform bioinformatics software Ugene v.1.21.0 (Okonechnikov *et al.*, 2012). Automated plasmid annotation was created in PlasMapper (Dong *et al.*, 2004). Manual analysis of sequences began with open reading frames (ORFs) prediction and gene function prediction using the GeneMark.hmm (Besemer and Borodovsky, 2005) and Rapid Annotations using Subsystems Technology (RAST) (Aziz *et al.*, 2008). Gene content was further ascertained by Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990), UniProt (UniProt, 2015) and Pfam (Finn *et al.*, 2014). Figures of sequence alignments were generated using EasyFig v.2.1 (Sullivan *et al.*, 2011).

3.22 Computational analysis of the SCC elements

Sequences of SCC elements were downloaded from the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). The borders of SCC elements were determined either from the annotation of the sequence or were established by the identification of the direct and inverted repeats in the attachment site of the *orfX* gene. Composite islands in *Staphylococcus saprophyticus* strain ATCC 15305 (Accession number AP008934) and *S. aureus* strain WAMRSA40 (Accession number JQ746621) were divided into separate SCC elements. SCC*mec* elements in *S. aureus* strain M1, M08/0126, and Sa0059 were included in the analysis with the Arginine Catabolic Mobile Element (ACME).

MLST types of strains analysed in this study were obtained from pubmlst.org (Jolley and Maiden, 2010) and http://saureus.mlst.net/ (Enright *et al.*, 2000).

SCC sequences were aligned using Mauve v.2.3.1. with the progressive Mauve algorithm (Darling *et al.*, 2010). Mauve identifies Locally Collinear Blocks (LCB) that are separately aligned in a specific format, so a custom-designed script was used to concatenate all defined corresponding blocks and to create a fasta file suitable for most of the multiple sequence alignment programs. Afterwards, the sequences were realigned using Mafft v.7.158 with the auto- method (Katoh and Standley, 2013). For evolutionary model prediction, the JmodelTest2 (Darriba *et al.*, 2012; Guindon and Gascuel, 2003) software was used with the Akaike Information Criterion (AIC) test setting. As the best model, the program predicted GTR + gamma + inv; hence the GTR + gamma model was employed when constructing the maximum-likelihood tree using Randomized Axelerated Maximum Likelihood (RAxML) v.7.2.8. (Stamatakis *et al.*, 2007). Node support was evaluated using 5000 bootstraps cycles.

4 Results

4.1 Identification of phages of *S. sciuri* and *S. petrasii*

For the identification of viable phages in the species *S. sciuri* and *S. petrasii* a new screening method based on UV-reactivation of prophages on a 96-well tissue culture plate was designed. Similar approach used McDonald and colleagues when inducing prophages from *E. coli* (McDonald *et al.*, 2010). If lysis zones were detected in the initial screening, the phages were reactivated in bigger volumes either by UV-light or mitomycin C. After the reactivation the phages were propagated from one plaque to obtain pure lysate.

Protocol of phage reactivation on 96-well plate was tested with *S. aureus* strain COL, whose reactivated phage is known to grow on *S. aureus* RN4220. Volume of the input bacterial suspension, incubation times and UV-dose were optimized to get the highest phage titter after the reactivation.

15 strains of *S. sciuri* (P583, P569, P548, P607, P612, P553, P581, P590, P575, P879, P611, P589, P602, P543, and P723) and 9 strains of *S. petrasii* (P5402, 5404, 5410, 5411, 5413, 5415, 6317, 6378, and CCM8494) were selected for the initial screening for temperate phages on the plate. The presence of the phages and their host range were tested on bacterial strains listed in Table 3 by the soft agar spot assay. Small plaques, matt plaques or lysis zones were produced by lysates from several strains as listed in Table 6. No lytic zones were observed in the bacterial lawn when testing CoNS phages on *S. aureus* RN4220.

Lysate	Susceptible strain	Comment
S. sciuri P879	*S. sciuri* P723	clear plaques
	S. sciuri P583, P612	rare matt plaques
S. sciuri P575	*S. sciuri* P723, P548	few distinct plaques
	S. sciuri P612	many distinct plaques
S. sciuri P581	*S. sciuri* P602, P569, P589	very matt lysis
	S. sciuri P607, P543, P553, P590	matt lysis
	S. sciuri P583	distinct plaques
S. petrasii P5404	*S. petrasii* P6317, P6318, P6320	matt lysis
	S. petrasii P5413	matt plaques
	S. petrasii P5402, P5411	distinct plaques
S. petrasii P5410	*S. petrasii* P5402	matt lysis
S. petrasii P5415	*S. petrasii* P6317	matt lysis

Table 6: Strains with possibly reactivated prophages, susceptible strains and characteristics of the lysis zones

The strain *S. petrasii* P5401 poured in the soft agar with 0.002M $CaCl_2$ on the MPA was not growing confluently; it is possible that some prophages from this strain were self-reactivating.

The strains *S. sciuri* P879, P575, and P581 and *S. petrasii* P5401, P5404, P5410, and P5415 with possibly reactivated phages were selected for further experiments. Firstly, phages from these strains were reactivated with UV-light and suitable propagation strains for obtained phages were selected according to the potential of the phages to lyse the host bacteria (Table 7). UV-induction did not confirm the presence of reactivable prophages in *S. petrasii* P5401, P5410, and P5415.

Species	**Phage**	**Propagation strain**
S. sciuri	ϕ575	P612
	ϕ581	P583
	ϕ879	P723
S. petrasii	ϕ5404	P5402 and P5411

Table 7: *S. sciuri* and *S. petrasii* phages and their propagation strains

Secondly, the phages were propagated from one plaque to obtain pure lysate. Phages ϕ879, ϕ575, and ϕ581 were not growing in broth, though several cultivation media (MPB, BHI) and approaches (cultivation in aerator or Erlenmeyer flask with shaking) were tested. Therefore, these phages were cultivated on plates. The highest PFU counts (up to 10^9 PFU/ml) were obtained from 2YT agar poured over with 0.7% soft agar with bacterial strain and 2 mM $CaCl_2$. Phages from *S. sciuri* P879, P575, and P581 were also reactivated chemically with mitomycin C with approximately 10^5 PFU/ml.

Phage ϕ5404 grows in liquid media, though it demands careful treatment. OD_{600} of the propagation strain in the aerator has to be around 0.2 before the addition of the phage lysate and the incubation time in the aerator should not exceed one and half hour. The PFU count in aerators was reached in span 10^7–10^9 PFU/ml. The propagation of phages in Erlenmeyer flasks with shaking or on plates gave lower PFU counts.

DNA from phage ϕ5404 was isolated with Phage DNA Isolation Kit to characterize this new phage deeper as the phage genome is to be sequenced and annotated. Before DNA isolation particles the phage ϕ5404 were purified by isopycnic centrifugation through CsCl gradient.

4.2 Phage adsorption rate assay

As a preparation for the transduction experiments, it was examined whether some *S. sciuri* phage particles are attaching to *S. aureus* and *S. sciuri* hosts. Hence the attachments of the

phage ϕ575 on *S. aureus* RN4220 and the phage ϕ879 on *S. aureus* RN4220, *S. sciuri* P574 and P600 was assessed by the phage adsorption rate assay (Figure 3). The number of phage particles (PFU) were detected in the starting lysate and in the supernatants taken in 5, 10, 15, 20, 25, and 30 minute after the addition of the lysate to the bacterial strain. Phage PFU/ml was assessed by the soft agar plaque assay on propagation strain *S. sciuri* P612 for ϕ575 and *S. sciuri* P723 for ϕ879.

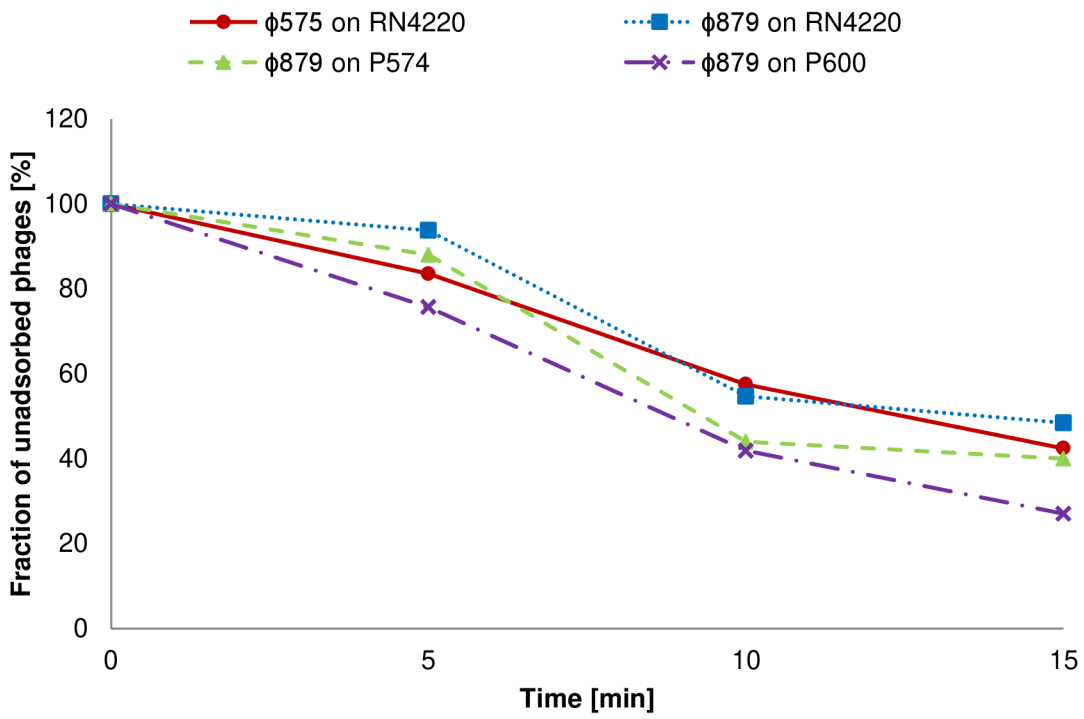

Figure 3: Adsorption of phages φ575 on strain *S. aureus* RN4220 and φ879 on *S. aureus* RN4220, *S. sciuri* P574 and *S. sciuri* P600 in 15 min interval. Samples were taken in time 0, 5, 10, and 15 min. The relative PFU/ml count is displayed as a mean of three replicas.

After the addition of the phages to bacterial culture, the relative PFU/ml count was declining slowly until fifteenth minute and later the relative phage count started increasing. The most phage particles were adsorbed around fifteenth minute in span 27 % for ϕ879 on *S. sciuri* P600 and 48 % for ϕ879 on *S. aureus* RN4220.

4.3 Characterization of bacterial strains with prophages and strains susceptible to phages

The sub-inhibitory concentration of tetracycline was determined for the *S. sciuri* strains P575, P581, P723, and P879 and *S. aureus* RN4220 in the antibiotic medium. The sub-inhibitory concentrations assessed from a steep decrease in OD_{600} are summarized in Table 8. The sub-inhibitory and inhibitory concentration of cadmium was determined on plates for the

S. sciuri strains P575, P581, P612, P723, and P879 and *S. aureus* 07/759 (Table 8). The only tetracycline-resistant strain was *S. sciuri* P723 as confirmed by disk diffusion antibiotic susceptibility testing in the NRL/St.

Strain	Tetracycline sub-inhibitory concentration (µg/ml)	Cadmium sub-inhibitory concentration (µmol/l)	Cadmium inhibitory concentration (µmol/l)
S. sciuri P575	0,25	75	100
S. sciuri P581	0,25	75	100
S. sciuri P612	N.D.	25	50
S. sciuri P723	resistant	100	250
S. sciuri P879	0,25	resistant	resistant
S. aureus RN4220	0,50	N.D.	N.D.
S. aureus 07/759	N.D.	resistant	resistant

Table 8: The sub-inhibitory concentration of tetracycline and cadmium, the inhibitory concentration of cadmium. N.D. – not determined

4.4 Characterization of selected MGEs of CoNS

The content of plasmids in the *S. sciuri* strains P537, P554, P574, P575, P581, P583, P600, P612, P723, and P879, was investigated in this work. Plasmid DNA was isolated with NucleoSpin Plasmid Isolation Kit (Macherey-Nagel) and restricted with *Hin*dIII endonuclease. The plasmid DNA fragments were detected on agarose gel. Plasmids were found in *S. sciuri* P574, P723, and P879. Presumably, the plasmid in P723 (named p723) carries gene responsible for tetracycline resistance, which was investigated by sequencing of this element.

Plasmid DNA of *S. sciuri* P723 was sequenced using the Ion Torrent™ PGM in the Laboratory of Molecular Diagnostics (LMDM) by the Ion Torrent sequencing platform operative. The whole p723 plasmid sequence was retrieved after the alignment of sequencing data. The length of the plasmid DNA is 5667 bp, with the GC content 35.27 %. There were predicted 4 ORFs in PlasMapper (Dong *et al.*, 2004), which were deeper characterized manually by protein BLAST in NCBI and UniProt databases. During the sequence analysis two more hypothetical proteins were found on the plasmid (coding sequence (CDS) 5 and 6). These CDSs have low sequence homology to already described proteins, and thus unknown function. The origin of the replication was not found, hence working plasmid sequence ‘beginning’ was chosen between genes for tetracycline efflux MFS transporter and plasmid recombination protein. Genetic map of the plasmid is visualised in Figure 4 and identified genes are listed in Table 9.

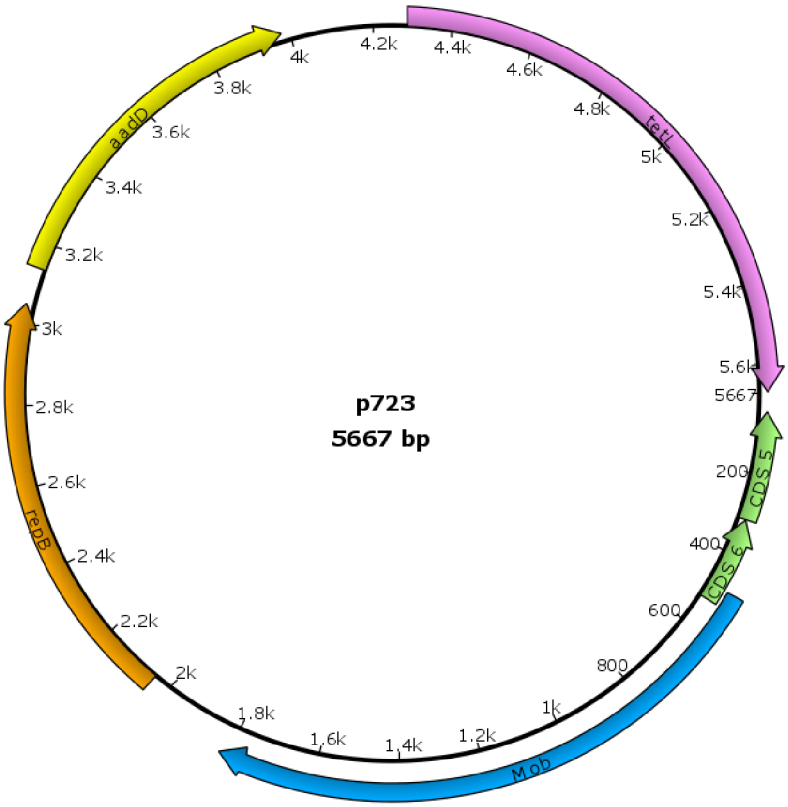

Figure 4: Genetic map of *S. sciuri* P723 plasmid p723. Mob – Plasmid recombination protein, RepB – Plasmid replication protein, AadD – Kanamycin nucleotidyltransferase, TetL – Tetracycline efflux MFS transporter, CDS – coding sequence. The figure was generated using UGENE bioinformatics software tool

Gene name	Start position	End position	Gene function
CDS 5	46	309	Hypothetical protein
CDS 6	312	521	Hypothetical protein
mob	483	1823	Plasmid recombination protein
repB	2047	3051	Plasmid replication protein
aadD	3142	3981	Kanamycin nucleotidyltransferase
tetL	4288	5664	Tetracycline efflux MFS transporter

Table 9: Annotation of plasmid p723 from *S. sciuri* P723

Based on available GenBank records, p723 is similar (99% identity) to several other plasmids from staphylococcal species: pKKS825 (Accession number FN377602), pUB110 (Accession number M19465), pERGB (Accession number JN970906) from *S. aureus*, SAP016A (Accession number GQ900381) from *S. epidermidis*, or pIP1714 (Accession number AF015628) from *Staphylococcus cohnii*. Plasmid p723 shows homology also to plasmids from enterococci, *E. coli*, and bacilli.

4.4.1 Computational analysis of SCC*mec* from *S. sciuri* P879

Whole genome sequencing of *S. sciuri* P879 was performed in the LMDM using the Ion Torrent™ PGM. After the sequence assembly and automated annotation with RAST (Aziz *et al.*, 2008), the contig with predicted *mecA* and *ccr* genes were manually inspected. The SCC*mec* found in this strain is probably type VII. It contains a class A *mec* gene complex (*mecA* – *mecR*1 – *mecI*), the *ccrB*3 gene closest (94% identity) to *S. sciuri* strain MCS24 (Accession number AB587080), and the *ccrA* gene closest (91% identity) to *ccrA*5 gene of *S. cohnii* strain WC28 (Accession number: GU370073) and *ccrA*1 gene (90% identity) of *S. sciuri* subsp. *carnaticus* strain GVGS2 (Accession number: HG515014). The SCC*mec* element was similar (98% identity) to SCC*mec* element type VII of *S. pseudintermedius* strain KM241 (Accession number: AM904731) and novel SCC*mec* element of *S. cohnii* strain WC28 (Accession number: GU370073), but their J1 and J3 regions were different (Figure 5). None of the direct or inverted repeat sequences at the borders of the element were detected.

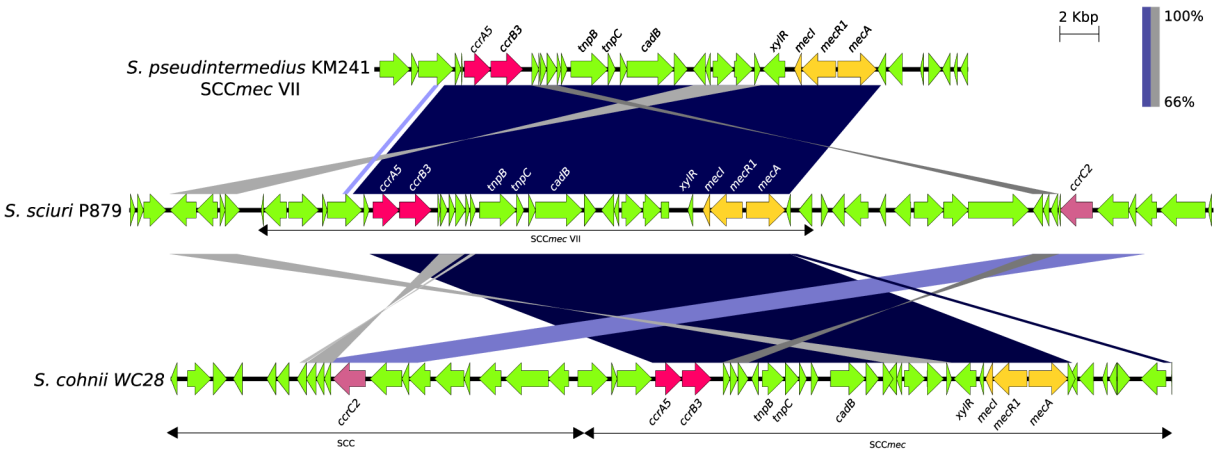

Figure 5: Comparison of SCC elements from *S. pseudintermedius* strain KM241 (Accession number: AM904731), *S. sciuri* strain P879 and *S. cohnii* strain WC28 (Accession number: GU370073). Positions and orientations of coding regions are represented by arrows. Conserved regions with more than 66% homology are indicated. The figure was generated using Easyfig v.2.1.

Another SCC element is possibly present on this contig as *ccrC* gene was found upstream of the SCC*mec* on this contig. Genes for type I RM system (*hsdM*, *hsdR* and *hsdS*), ABC transporter genes and genes for hypothetical proteins were found in between the *ccrC* gene and SCC*mec* element. The remnants of the *orfX* gene that contain the insertion site for SCC elements were not on this contig, partial sequence of the *orfX* was on another contig.

4.4.2 Detection and quantification of bacterial genes in phage particles by qPCR

Earlier, Zeman found parts of the P723 plasmid DNA and SCC*mec* while annotating the sequence of ϕ879 (Zeman, 2015). So, qPCR was used to prove the packaging of the *S. sciuri*

plasmid P723 and SCC*mec* in phage particles using methodology invented by Mašlaňová *et al.* (2013) with primers designed by Zeman.

Prior to the quantification experiment, the homogeneity and purity of the phage lysate was ensured by the purification of the phage particles in CsCl gradient and subsequent dialysis in phage buffer. The presence of exogenous bacterial DNA was excluded by detection of control DNA. In brief, control DNA of *B. breve* ATCC 15700 in 100× higher concentration then detection limit for genus-specific DNA (0.005 ng/µl) was added to the phage lysate followed by DNase treatment and DNA isolation. PCR and qPCR did not showed presence of any *B. breve* DNA, so obtained results show only amplification of DNA encapsulated in phage particles.

For the relative quantification analysis the plasmid-borne gene for kanamycin resistance (kanamycin nucleotidyltransferase) and part of the bacterial chromosome (SCC*mec* element) were chosen as target genes. Phage adaptor protein between head and tail specific for *S. sciuri* bacteriophages was used as the reference gene. The mean C_p values were obtained out of three replicas for the phage DNA undiluted, diluted 10× and 100×. C_p values for undiluted DNA were too low, thus biased so they were excluded. The calculated ratio between the number of target gene copies (SCC*mec* or kanamycin nucleotidyltransferase) and the reference gene copies (phage adaptor protein) is visualized in Figure 6. Obtained data suggest packaging of the bacterial genes in phage capsid. Plasmid-borne gene kanamycin nucleotidyltransferase was detected to be packaged more often (at least 1 000×) than the part of the bacterial chromosome, the SCC*mec*.

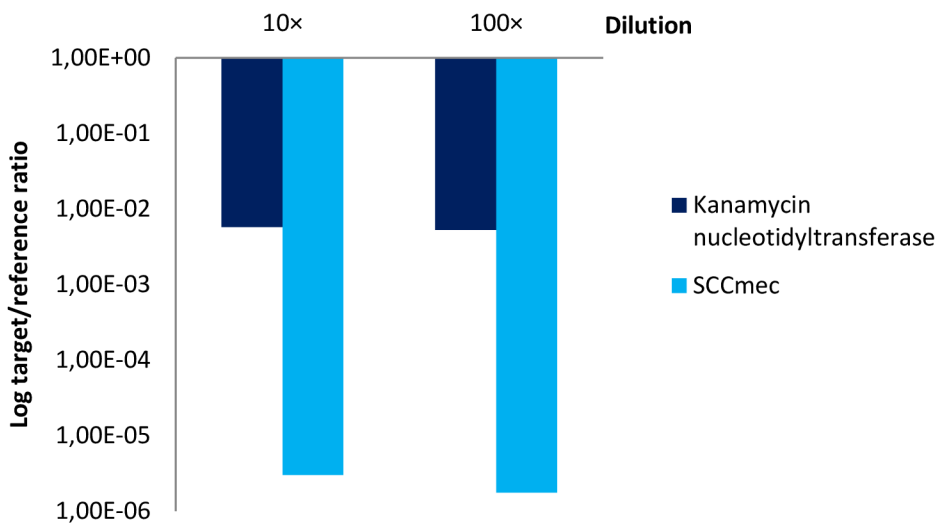

Figure 6: Relative quantification of kanamycin nucleotidyltransferase and SCC*mec* in phage particles

4.5 Transduction experiments

S. sciuri phages were tested for their potential to transduce bacterial genes. For this purpose it was necessary to prepare *S. sciuri* strains with plasmids that could be transduced and that enable selection of transductants. Two plasmids, tetracycline resistance plasmid pT181 from *S. aureus* strain COL and penicillinase/cadmium resistance plasmid pUSA300-HOUMR-like from *S. aureus* NRL/St/07/759, that were previously used in transduction experiments by Varga *et al.* (2012) were chosen to be introduced into *S. sciuri*.

For the introduction of the plasmids into the *S. sciuri*, the transformation by electroporation was employed. The electrocompetent cells were tried to be made from *S. sciuri* P575, P581, P879, P583, and P612. The restriction deficient *S. aureus* RN4220 was selected as control recipient strain. The competent cells were prepared according to two different protocols, the glycerol-based (Lee, 1995) and the sucrose-based (Kraemer and Iandolo, 1990). The glycerol based preparation of competent cells proved to be ineffective for all *S. sciuri* strains as transformants were obtained only from *S. aureus* RN4220. The second approach of competent cells preparation in sucrose was successful, and after the electroporation of the plasmid DNA, several transformants were obtained. The detection of transformants was done on plates with selection agent, whose concentration was determined in previous experiments, for tetracycline c = 5 µg/ml and cadmium c = 5 nM.

Presumed transformants *S. sciuri* P581::pT181, P612::pT181, P581::pUSA300-HOUMR-like, and P612::pUSA300-HOUMR-like and the transformants from positive control RN4220::pT181 and RN4220::pUSA300-HOUMR-like were subjected to several tests to

exclude possible contaminants. Firstly, plasmid DNA was isolated and analysed by RFLP, secondly plasmid DNA and cell lysate were analysed by PCR for detection of resistance genes *tetK* for pT181, and *blaZ* and *cadD* for pUSA300-HOUMR-like. Thirdly, macrorestriction profiles of recipient strains and transformants were compared. The testing revealed that transformants *S. sciuri* P581::pT181 and P612::pT181 were obtained in addition to positive control RN4220::pT181 and RN4220::pUSA300-HOUMR-like. *S. sciuri* P581 and P612 that were able to grow on plates with cadmium had similar macrorestriction profile to susceptible strains used as recipients for electroporation, but neither the PCR nor the RFLP detected any plasmid DNA. They probably acquired some chromosomal mutations responsible for cadmium resistance, hence they were not used for the transduction experiments.

Several transduction experiments were performed to assess the transduction potential of the *S. sciuri* phages. *S. sciuri* strains P574 and P600 were used as recipient strains, for they were found to be susceptible to *S. sciuri* phages. Apart from the *S. sciuri* strains, *S. aureus* RN4220 was used as a recipient for the transduction experiments, the setups are summarized in Table 10.

Donor	**Phage**	**Recipient**
S. sciuri P581::pT181	reactivated by mitomycin C	*S. aureus* RN4220
S. sciuri P612::pT181	φ575	*S. sciuri* P574
S. sciuri P723	φ879	*S. sciuri* P600

Table 10: List of donors, transducing phages, and recipients used for transduction experiments

Plasmid pT181 encoding the resistance to tetracycline was used as a selection marker. None of the recipient strains grows on tetracycline plates (c = 5 µg/ml) whereas all donor strains are growing on such tetracycline concentration. *S. sciuri* P723 has a plasmid presumably responsible for tetracycline resistance, so this plasmid was also subjected to transduction experiments.

Transducing lysates were prepared either by propagation of the phages ϕ575 on *S. sciuri* P612::pT181 and ϕ879 on *S. sciuri* P723 or by reactivation of the phage ϕ581 from *S. sciuri* P581::pT181 with mitomycin C. Then transduction approaches based on the protocols by Olson and Horswill (2014) and Krausz and Bose (2016) were tested. In the first experimental array the transducing lysate was mixed with recipient strain with different multiplicity of infection (MOI) 10; 5; 1; 0.2; and 0.1. In the second array the RM system of the recipient strains was inactivated by high temperature as follows: bacterial culture was concentrated by

centrifugation (5 000 rpm, 10 min) and resuspended in 1 ml of MPB. 60 µl of this heavy suspension was added to 2 ml of MPB previously warmed to 56°C. This temperature was maintained for 2 minutes after which the tubes were rapidly cooled and the suspension was mixed with the lysate. Potential transductants P600::pT181 and P574::pT181 were obtained after the selection on tetracycline plates.

The transduction was verified by the analysis of the suspected transductants using plasmid DNA restriction analysis, PCR for detection of resistance gene *tetK* and PFGE. Plasmid analysis revealed that all of the possible transductants contain a plasmid, but it was not the pT181 plasmid as verified by the restriction analysis with *Apa*I, *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Not*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I and the PCR results. Macrorestriction profiles of possible transductants were similar to macrorestriction profiles of recipient strains used for the transduction. In conclusion, the transduction ability of the phages was not proven. There may be several possible reasons, why the transduction was not observed: i) the plasmids cannot be stably maintained in recipients, ii) the frequency of transduction is too low to be sighted, iii) the phages are unable to deliver the plasmid in a cell, or iv) the RM system of the recipient cleaves plasmid DNA.

4.6 Phylogenetic analysis of the SCC elements

Maximum likelihood phylogenetic tree (Figure 7) was constructed for 81 SCCs downloaded from the NCBI GenBank Database. The crucial step of the analysis was the alignment of the SCC element sequences. Most of the computational tools were unable to create an alignment, but Mauve did. The whole sequences were divided by Mauve into LCBs, which determined the related regions of the SCC elements. The LCBs of each SCC sequence were concatenated and the concatenate was used for multiple alignments. The best evolutionary model was predicted GTR + gamma + inv according Akaike Information Criterion (AIC) test setting. Due to the quantity of the data, only RAxML (Stamatakis *et al.*, 2007) was evaluated as the best program for phylogenetic tree construction. The computation with other used programs MrBayes (Ronquist and Huelsenbeck, 2003) and PhyML (Guindon *et al.*, 2010) was extremely slow, despite the process parallelization and usage of 12 CPUs/60 GB of RAM.

The maximum likelihood phylogenetic tree clustered the same types of SCC*mec* in distinct clades showing that SCCs share an essential part of genetic information. The shared genes are the *mec* and *ccr* gene complexes. The topology of the phylogenetic tree does not indicate any association between ST (CC) and SCC*mec* type.

In the analysed data set, SCC*mec* I elements showed a quite close phylogenetic relationship, although their sequences were from two clonal complexes, CC5 and CC8. Similarly, clusters of SCC*mec* II and SCC*mec* III look quite homogeneous with high bootstrap support. SCC*mec* IV elements were the most abundant in the data collection, disseminated in nearly all clonal complexes. Despite their diverse origin, SCC*mec* IV elements formed a compact cluster in the phylogenetic tree with high confidentiality, which is a good evidence of the tree stability. SCC*mec* V elements were ranged in a separate branch apart from other SCC elements, closely related to SCC*mec* VII. Sequences of SCC*mec* types VI, VII, VIII, IX, X, and XI, though of limited count, were included in the analysis to obtain more precise results and to ascertain their evolution. In the analysis, they grouped with other *SCCmec* types with similar *mec* and *ccr* gene complexes. The non-*mec* SCC elements such as ψSCC_{12263}, ψSCC_{476}, and ψSCC_{45394} and SCC carrying various genes like SCC*sorbitol*, SCC_{cap} (capsule), SCC_{15305}RM (restriction-modification), SCC*pls* (plasmin), and SCC*pbp4* (penicillin-binding protein 4) were in separate clusters and quite distantly related to SCC*mec*.

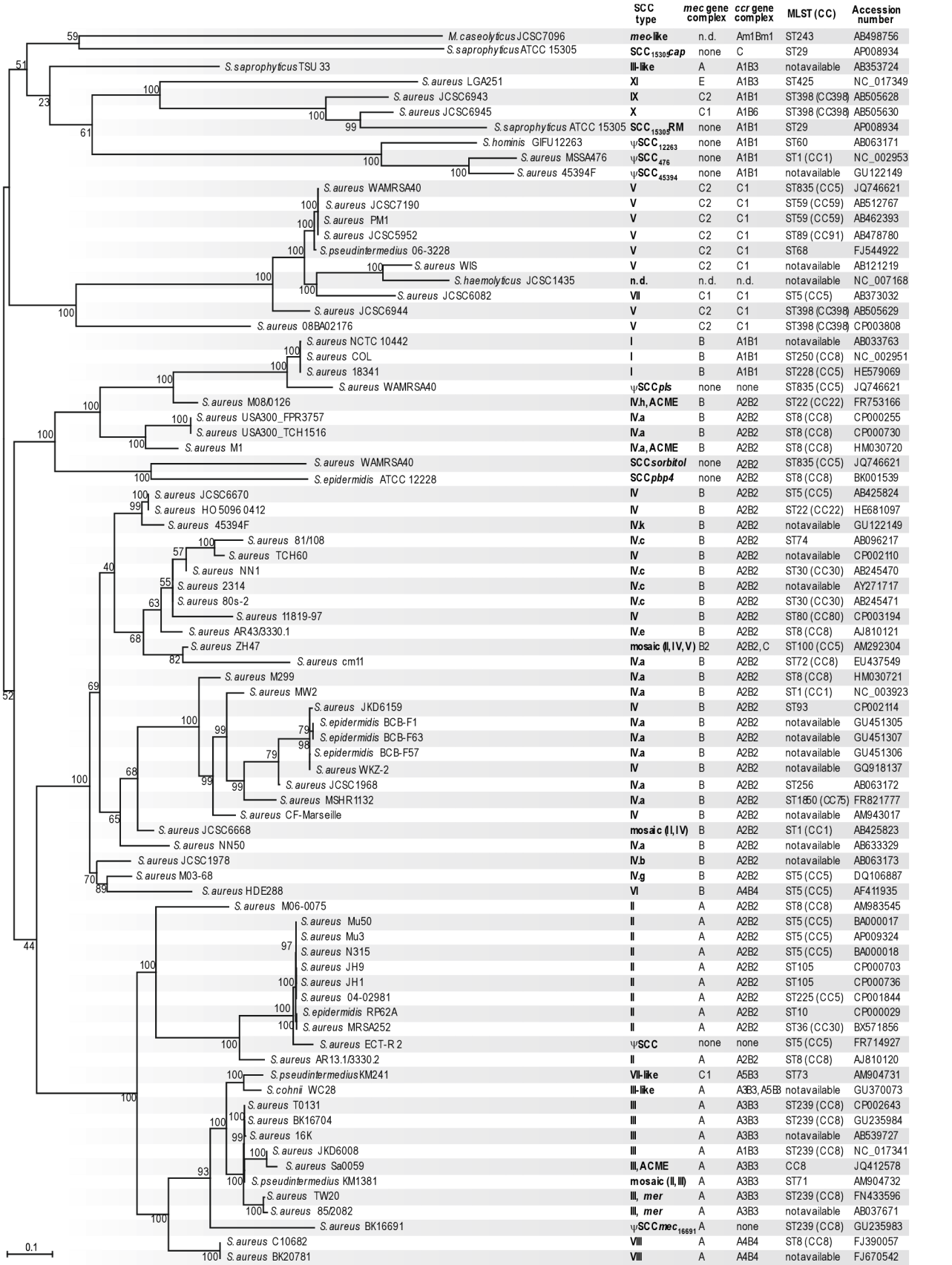

Figure 7: Unrooted maximum likelihood tree showing phylogenetic relationships among SCC elements. The phylogenetic tree was inferred using the GTR + gamma nucleotide substitution model. Node support is calculated from 5000 bootstrap cycles. The scale bar indicates an evolutionary distance of 0.1 nucleotides per position in the sequence. n.d. – not determined.

5 Discussion

Bacteria adapt quickly to changes in the environment. They have extremely variable genomes as illustrated for staphylococci whose substantial part of the genome (around 25 %) is composed of MGEs (Lindsay, 2014). The genome dynamics and plasticity is vastly shaped by the HGT with the biggest impact on the spread of virulence and resistance determinants. Important mediators of the HGT in staphylococci are phages. In this work, **four new phages** of CoNS were discovered, ϕ575, ϕ581, and ϕ879 infecting *S. sciuri* and ϕ5404 infecting *S. petrasii*. To our knowledge, these viruses are the very first described phages infecting *S. sciuri* and *S. petrasii* species. ϕ575 and ϕ879 were characterized deeper in M. Zeman's diploma thesis (2015). Additional characterization of phages and their propagation strains was done as a part of this work.

The new CoNS phages were identified using completely new custom-made approach of **phage induction on 96-well plate.** Similar approach of phage detection was developed for Gram-negative bacteria (McDonald *et al.*, 2010), but there were no protocols available for staphylococci. The 96-well plate-based method enables examination of many specimens while reducing the handling time and usage of material. This optimized screening method proved to be effective for detection of viable temperate phages of *S. sciuri* and *S. petrasii* in this work, and it was already successfully tested on *S. epidermidis* in our laboratory.

Phage fitness is determined by three phenotypes: adsorption rate, burst size and lysis time (Bull *et al.*, 2011). In this work, **adsorption rate** of ϕ575 and ϕ879 was examined. Adsorption rate of these CoNS phages on *S. aureus* RN4220 and *S. sciuri* strains P600 and P574 was very slow, compared to other phages such as ID11 infecting *E. coli* (Roychoudhury *et al.*, 2014) or SE2 infecting *Salmonella enterica* (Tiwari *et al.*, 2012). The number of PFU/ml in tested lysate was decreasing constantly and most phages were cleared out of the lysate after 15 minutes, whereas the majority of the ID11 phage particles were adsorbed in first three minutes, or most of the SE2 phage particles in first 6 minutes. Additionally, the fraction of unadsorbed phages in tested lysate stayed very high, up to 48 % for ϕ879 tested on *S. aureus* RN4220 in contrast to phage ID11, where the unadsorbed fraction was ranging around 10 % or phage SE2 which adsorbed completely after 20 minutes. Complete adsorption of the *S. sciuri* phages was not observed, after prolonged incubation time the PFU/ml started increasing, possibly due to the release of the phage progeny. Phages with slower adsorption rate have slower infectivity, which might be beneficial for phage fitness (Gallet *et al.*, 2011; Roychoudhury *et al.*, 2014), but the very slow adsorption of tested phages together with high

number of unabsorbed phages might suggest only rare attachment of the phages on cell surface.

Phage attachment is a key step in a delivery of the DNA into the host cell. Phages attach via phage receptor binding protein to its bacterial cell surface receptor (Samson *et al.*, 2013). Bacterial receptors are usually surface proteins, polysaccharides and lipopolysaccharides (Rakhuba *et al.*, 2010). Staphylococcal phages employ residues of bacterial wall teichoic acid for their attachment (Xia *et al.*, 2011). While *S. aureus* produces wall teichoic acid with ribitol-phosphate modified with α- and/or β-linked GlcNAc and D-Ala, various CoNS produce wall teichoic acid composed of glycerophosphate with different types of sugars (Weidenmaier and Peschel, 2008). The specificity of the phage attachment to *S. aureus* and CoNS can thus differ and it is not clear whether CoNS phages attach to *S. aureus* host. Obtained data on phage adsorption might imply only weak interactions between the phage tail adhesins and bacterial wall. Such unspecific interactions may represent an obstacle for transfer of the DNA into the host cells (Bebeacua *et al.*, 2013).

Genomes of phages ϕ575 and ϕ879 were sequenced and annotated in M. Zeman's diploma thesis (2015). Phage ϕ5404 DNA was isolated as a part of this work and is to be sequenced. Also, purified phage ϕ5404 particles are going to be sent for electron microscopy to ascertain the taxonomy of the phage. Based on the current knowledge, it should most probably belong to order *Caudovirales* and family *Siphoviridae*, because temperate staphylococcal phages were so far categorized as siphoviruses (Xia and Wolz, 2014). Prophage ϕ5404 in its host *S. petrasii* P5404 may strongly influence the bacterial phenotype. In our laboratory, several *S. petrasii* species have been subjected to PFGE and interestingly, the P5404 was the only strain indigestible by *Sma*I restriction enzyme. *Sma*I is sensitive to DNA methylation, so it is possible that prophage ϕ5404 carries a gene for DNA methyltransferase most probably as a part of some RM system as earlier reported by Dempsey and collective for *S. aureus* phage π42 (2005). This hypothesis will be proven after the inspection of the phage ϕ5404 genome sequence.

Another inspected trait of *S. sciuri* species was their **plasmid** content. Plasmids were detected in strains P574, P723, and P879. Plasmids substantially affect the antibiotic sensitivity of their carrier strains (Kuntová *et al.*, 2012). Indeed, the tetracycline-resistant *S. sciuri* P723 has a plasmid which encodes one of the tetracycline efflux genes as detected by sequence analysis of this element. Plasmids in *S. sciuri* P879 and P574 were not studied in detail, but in the future it will be interesting to find out, what their gene composition is.

The sequencing of the p723 plasmid confirmed that tetracycline resistance in the P723 is encoded by plasmid-borne gene *tetL*. Additionally, the structural analysis of the p723 plasmid identified *aadD* gene responsible for resistance to kanamycin and neomycin. Similar gene content as the p723 plasmid have several other staphylococcal as well as non-staphylococcal plasmids. Such sequence homology could be explained by interplasmid recombination, which leads to derivation of new plasmids (Schwarz *et al.*, 1996).

Detection and **relative quantification** of resistance genes from *S. sciuri* plasmid p723 in phage particles was performed using qPCR. Phage ability to package bacterial genes was also tested for the chromosomal SCC*mec*. The packaging of bacterial DNA was confirmed for both elements; the relative quantity of the plasmid p723 DNA was higher, than the relative quantity of SCC*mec* region. This result may imply that p723 plasmid is packaged as a multimer as suggested for other small plasmids (Novick *et al.*, 1986) or the plasmid is simply getting more often into the phage capsids. Substantial increase in plasmid packaging was observed by Novick and collective (2007) when cloned fragment of phage ϕ11 DNA was inserted into the plasmid sequence, but during the sequence analysis of the p723 plasmid no phage genes were identified.

New SCC*mec* element was found in *S. sciuri* P879 during analysis of data from sequencing of this strain. The study of SCC*mec* in the *S. sciuri* brings another evidence of diversity of these elements and reveals the gene pool associated with the methicillin resistance. SCC*mec* was characterized to be type VII, carrying a class A *mec* gene complex, with new variants of *ccrA*5 and *ccrB*3 recombinases. The sequence analysis also revealed that this SCC*mec* is probably arranged in tandem with additional SCC element with *ccrC*. The two elements might have integrated independently or they can constitute a composite as speculated for SCC elements in *S. cohnii* strain WC28 (Zong and Lu, 2010). The SCC*mec* element is bordered by repetitions, inverted of length 27 bp and direct of length 15 bp (Katayama *et al.*, 2000). The neither direct nor inverted repeats were found flanking the SCC*mec* element from *S. sciuri* P879. Sometimes the repeats are not present as reported for *S. aureus* strain cm11 (Park *et al.*, 2009). Another reason for the difficult identification of the repeats could be the fact that this chromosomal region can contain a composite island built from more SCC elements. In future, it will be necessary to design primers to obtain rest of the sequence between *orfX* and the end of the contig with the SCC*mec* and look for the repeats in the currently missing piece.

The ability of the ϕ575 and ϕ879 to transfer bacterial DNA was tested in several intra- and interspecies transduction experiments. Phage propagation strains and lysogens with phages

were firstly modified by **transformation** to enable controlled experimental approach. The *S. sciuri* strains were subjected to electroporation of two plasmids: the tetracycline resistance plasmid pT181 and the penicillinase/cadmium resistance plasmid pUSA300-HOUMR-like. After the electroporation two *S. sciuri* transformants P581::pT181 and P612::pT181 were obtained. The only effective method for the *S. sciuri* electro-competent cell preparation turned out to be based on sucrose decreasing concentration as designed by Kraemer and Iandolo (1990). The reason for that might be the different composition of the cell wall of *S. aureus* and CoNS (Xia *et al.*, 2011). None of the *S. sciuri* strains with plasmid pUSA300-HOUMR-like were prepared by electroporation. This larger (27 kb) plasmid is more difficult to wedge in the bacterium than the smaller ones such as the 4.4 kb pT181 plasmid. Regrettably, the electro-competent cell preparation in glycerol as described by Lee (1995), which yields higher frequencies of transformants with larger plasmids, was not effective in *S. sciuri*. Transformation also requires overcoming of RM-barriers, namely type IV RM system and type I RM system (Corvaglia *et al.*, 2010; Xu *et al.*, 2011), so it is possible that other transformants were not obtained due to the restriction of the DNA.

The **transduction** ability of the newly discovered CoNS phages ϕ575, ϕ581, and ϕ879 was not detected though several experimental approaches in numerous replications were tried. There may be many reasons, why the transduction was not observed as there are many critical steps in the transduction process as the DNA packaging, DNA delivery inside the recipient cell and fate of the DNA inside the cell.

Firstly, phages have to able to pack the bacterial DNA inside their capsids. pT181 plasmid, which was proved to be packaged by various phages (Chlebowicz *et al.*, 2014) and which was effectively used in transduction among *S. aureus* (Varga *et al.*, 2012), was chosen for the transduction experiments. Additionally, the ability of the new CoNS phages to carry bacterial DNA inside their capsids was detected by relative quantification experiments in this work. Therefore, it was assumed that pT181 is packaged inside the capsids. PCR and qPCR for detection of pT181 specific locus could verify this hypothesis.

Secondly, phages have to attach to the recipient and deliver the DNA inside the cell. The phages infect *S. sciuri* P574 and P600, but infection of *S. aureus* RN4220 was not productive. However, the phage sensitivity shall not be requirement for transduction (Chen and Novick, 2009), it is important if phages are adsorbing to the cells. In this work, the adsorption rate assay showed that some phage particles are attaching to the *S. sciuri* P574 and P600 and *S. aureus* RN4220 strains. If the phages are interacting with the bacterium only non-

specifically, it may prevent DNA from entering the recipient cell (Bebeacua *et al.*, 2013). The mechanism of DNA entry into the bacterium is still a poorly understood process. Potentially some virion proteins with murolytic activity are involved in peptidoglycan digesting during virus entry (Kivela *et al.*, 2004; Paul *et al.*, 2011). Such proteins might show specificity for cell walls of distinct species and since CoNS and *S. aureus* exhibit altered glycosylation (Winstel *et al.*, 2013; Xia *et al.*, 2011) it may also prevent HGT. Detailed structural and functional studies of phage parts should enlighten this matter in future.

Thirdly, the transduced DNA must not be cleaved by the RM system of the host, it must be stably maintained in the cell, and the selection marker has to be expressed. To avoid the cleavage of the DNA the chemically mutagenized *S. aureus* strain RN4220 with non-functional *hsdR* genes (Waldron and Lindsay, 2006) is used as recipient in transduction experiments. CoNS, particularly *S. sciuri* strains P574 and P600, have uncharacterized RM systems, which may cause the loss of the transduced DNA.

Finally, other factors, such as the transduction frequency, are important in the assessment of the phage transduction ability. If the transduction frequency is very low, it may be difficult to capture the transductants. Also, behaviour of the system might substantially differ inside laboratory and outside, the HGT could be more intensive *in vivo* (McCarthy *et al.*, 2014). The importance of the factors influencing transduction definitively varies for each species used in the experiment and in the future, it will be important to focus on the RM systems of the recipients, whether they are able to receive the plasmids or try to use other recipient strains.

As a part of this work the phylogenetic tree was constructed using whole **SCC sequences**. Earlier, Lina *et al.* (2006) have inferred the evolution of SCC*mec* from *ccr* complex genes and similarly, Urushibara *et al.* (2011), Rolo *et al.* (2014), and Park *et al.* (2013) have focused on *ccrA* and *ccrB* genes, however in this work, the whole SCC sequences were taken for the *in silico* analysis.

The SCC clustering did not reflect the genetic relationships of bacteria containing the SCC elements as SCCs from different STs (CCs) were dispersed in several clusters. This may be significant evidence for the introduction of SCC*mec* into some ST (CC) on several occasions as suggested by the evolutionary models of MRSA emergence by Robinson and Enright (2003). It is also possible that strains from one ST (CC) can lose a SCC*mec* and acquire another one (Diep *et al.*, 2006). The presence of similar SCC*mec* I elements in divergent genotypes seems to be a consequence of HGT (Enright *et al.*, 2002). Comparably, SCC*mec* IV was conceivably repeatedly introduced into different STs (CCs), as evidenced for CC8.

SCC*mec* IV elements from CC8 in the analysis were more distantly related to each other than to SCC*mec* IV from other CCs. The acquisition of SCC*mec* IV is facilitated mainly by its small size (20 – 35 kb) (Robinson and Enright, 2003).

SCC elements were grouped based on the combination of *mec* gene complex class and *ccr* gene complex type, which reflects the SCC*mec* classification well. Since SCC elements have unique mosaic structures, their evolution is dependent on large scale element rearrangements, including loss and gain of other MGEs (like plasmids and insertion sequences). The capture of other resistance and virulence determinants is promoted by the selection pressure (Robinson and Enright, 2003). Antibiotic usage in healthcare facilities enhances mainly the evolution of SCC elements carried by nosocomial pathogenic staphylococci. SCC*mec* I, II, and III are commonly isolated form HA-MRSA. These elements can accumulate several MGEs so they can reach lengths up to 80 kb. Such long elements can be a fitness burden for bacteria (Ender *et al.*, 2004; Knight *et al.*, 2013; Lee *et al.*, 2007), therefore clones with smaller SCC*mec* are becoming predominant nosocomial pathogens. Probably the most prevalent type of SCC*mec* in MRSA is SCC*mec* IV (Daum *et al.*, 2002) and clones with SCC*mec* IV are also becoming predominant nosocomial pathogens, as is the case of MRSA strains from ST22 (CC22) (Albrecht *et al.*, 2011; Kinnevey *et al.*, 2014; Knight *et al.*, 2012)

The phylogenetic tree topology also reveals that SCC elements with *ccrC* genes, SCC*mec* V and SCC*mec* VII, are closely related. The independent evolution of SCC*mec* V and SCC*mec* VII may have been due to the specificity of CcrC recombinase (Ito *et al.*, 2004). Several *ccrC* genes were identified in chromosomes of CoNS species; therefore, SCC*mec* elements might have emerged in one of the CoNS species. Candidate species for the creation of SCC*mec* V could be *S. haemolyticus* (Bouchami *et al.*, 2012), *S. saprophyticus* (Kuroda *et al.*, 2005), or *S. pseudintermedius* (Black *et al.*, 2009; Descloux *et al.*, 2008). The SCC*mec* V transfer from a CoNS species into *S. aureus* could be frequently happening among staphylococci inhabiting animals, since many SCC*mec* V elements were found in *S. aureus* strains CC398, predominantly associated with animals (Argudin *et al.*, 2010; Price *et al.*, 2012). Analogously, CoNS are reservoirs of other SCC*mec* types. SCC*mec* IV might have been introduced into *S. aureus* from *S. epidermidis* as suggested by Barbier *et al.* (2010) and results from the analysis. SCC*mec* IV.a elements from three isolates of *S. epidermidis* strain BCB were closely related to SCC*mec* IV.a from S. *aureus* strain WKZ-2 in the phylogenetic study.

The results unambiguously support the hypothesis that the SCC element is horizontally transferred probably across the species barrier, since the SCC elements from different species

cluster together. A plausible transfer mechanism seems to be the phage mediated transduction (Chlebowicz *et al.*, 2014; Scharn *et al.*, 2013). SCC*mec* or a part of SCC are packaged into phage capsids (Mašlaňová *et al.*, 2013) and if such DNA is injected into the recipient bacterium already carrying an SCC element, new SCC types might be created via recombination (Rolo *et al.*, 2012). Also mutations (Lina *et al.*, 2006) and insertion/excision of the *mec* or *ccr* gene complexes in diverse SCC types (Hanssen *et al.*, 2003) are playing a role in the evolution of SCC. The SCC evolution differs among species of the genus *Staphylococcus*. Some species, i.e. *S. aureus*, harbour the SCC elements irregularly, whereas in other species, i.e. *S. sciuri*, these elements are found in the majority of isolates, so CoNS may represent a potential reservoir for SCC elements (Otto, 2013).

6 Summary

Dissemination of virulence and resistance factors in staphylococcal population remains a threat for the treatment of diseases caused by staphylococci. Serious problems particularly for immunocompromised patients are the infections caused by *S. aureus* as well as CoNS. In recent years, the CoNS are being recognized as a possible source of the genes encoding the resistance factors. Such genes are often part of the MGEs and can be thus quickly spreading via HGT.

To understand the role of the HGT in the evolution of genus *Staphylococcus* the MGEs of CoNS were investigated. Both the HGT and the species evolution are tightly linked to phages, so a new screening method for detection of phages was designed and implemented. Using the new 96-well-plate-screening method three new phages of *S. sciuri* (ϕ575, ϕ581, and ϕ879) and one new phage of *S. petrasii* (ϕ5404) were discovered. The cultivation conditions, host range, and adsorption rate of the phages were analyzed in this work. The ability of phage ϕ879 to package bacterial genes was confirmed by the qPCR.

Major part of the experimental work was focused on the assessment of transduction abilities of the phages ϕ581 and ϕ879. Despite several optimizations of the transduction conditions, the transduction ability of the phages ϕ581 and ϕ879 was not proven, but it cannot be ruled out. The preparation for the transduction experiments included the transformation of the *S. sciuri* species based on electroporation. The suitable method for electro-competent cells preparation was found to be based on sucrose decreasing concentration and *S. sciuri* strains P612 and P581 with plasmid pT181, which can be utilized in other experiments, were prepared.

Furthermore as part of this work several strains of *S. sciuri* were deeper characterized, their antibiotic susceptibility was tested and content of plasmids was investigated. The plasmid p723 from *S. sciuri* P723 was sequenced and annotated. SCC*mec* element type VII was found during sequence annotation of *S. sciuri* P879. In addition, the phylogenetic tree for 81 SCC elements was constructed and the SCC evolution was discussed.

The results confirm that CoNS contain a myriad of MGEs that increase variability of the gene pool in staphylococcal population. The crucial discovery of the new CoNS phages is implying that phages are common among staphylococci, so they can represent the interplay between HGT and the evolutionary dynamics of the genus *Staphylococcus*.

7 Souhrn

Rozšiřování virulenčních a rezistenčních faktorů v populaci stafylokoků představuje neustálou hrozbu při léčbě infekcí způsobených stafylokoky. Velkým problémem hlavně u imunokompromitovaných jedinců jsou infekce způsobené nejen *S. aureus*, ale také koaguláza-negativními stafylokoky (KNS). V posledních letech navíc přibývá důkazů, že právě KNS jsou důležitým zdrojem genů zodpovědných za rezistenci k antimikrobiálním látkám. Rezistenční a virulenční determinanty, které se zpravidla nachází na MGE, jsou nejrychleji vyměňovány mezi bakteriemi prostřednictvím horizontálního přenosu genů.

V rámci práce byly studovány MGE u KNS, jež ukazují na roli horizontálního přenosu genů v evoluci rodu *Staphylococcus*. Evoluce druhů stafylokoků a HGT jsou úzce spjaty s fágy, tudíž první část práce byla zaměřena na rychlou detekci bakteriálních virů. Byla navržena a optimalizována metoda detekce fágů na 96-jamkové kultivační destičce, díky níž byly objeveny tři nové fágy u druhu *S. sciuri* (ϕ575, ϕ581 a ϕ879) a jeden nový fág u druhu *S. petrasii* (ϕ5404). V rámci práce byly také zjištěny optimální podmínky pro množení fágů, jejich spektrum hostitelů a rychlost adsorpce. Dále u fága ϕ879 byla metodou qPCR ověřena jeho schopnost sbalovat do fágových kapsidů bakteriální geny.

Značná část experimentální práce byla zaměřena na zhodnocení schopnosti nově objevených fágů ϕ581 a ϕ879 přenášet bakteriální geny transdukcí. Přestože byly vyzkoušeny různé podmínky pro transdukční experimenty, transdukční schopnosti ani jednoho ze zkoumaných fágů nebyly potvrzeny. Při rámci přípravy transdukčních experimentů byly transformovány *S. sciuri* prostřednictvím elektroporace. Bylo zjištěno, že elektrokompetentní buňky *S. sciuri* lze připravit podle protokolu založeného na snižující se koncentraci sacharózy. Plazmid pT181 byl úspěšně elektroporován do *S. sciuri* kmene P612 a P581 a takto upravené lze využít v dalších experimentech.

Dále bylo v práci charakterizováno několik kmenů *S. sciuri*, byla zjištěna jejich citlivost na antibiotika a detekován obsah plazmidů. Plazmid p723 z kmene *S. sciuri* P723 byl osekvenován a anotován. Analýzou sekvenčních dat byl také objeven SCC*mec* element typu VII u kmene *S. sciuri* P879. V rámci práce byl sestrojen fylogenetický strom z 81 SCC a na základě této analýzy byly shrnuty evoluční vztahy těchto elementů.

Získané výsledky potvrzují, že KNS obsahují plejádu MGE, které navyšují zásobu genů v populaci stafylokoků. Nejvýznamnějším výsledkem je objev nových fágů u KNS, který prokazuje, že fágy jsou běžnou součástí bakteriálního světa a představují tudíž významný mezičlánek mezi HGT a evolucí stafylokoků.

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