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Allelic variation in sdrD among Staphylococcus aureus from healthy carriers and its role in adhesions

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Summary

S. aureus is aerobic, catalase-positive, and oxidase negative organism, and present in the normal microbial flora of the upper respiratory tract and the skin without causing any disease, but at the same time as a potential humans pathogen. *S. aureus* adheres to the host tissues by means of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).

SdrD is one of three-sdr family proteins, which plays a role in adherence to human nasal epithelial cells. It is assumed that the A-domain of SdrD interacts with a host protein, which is not yet identified.

The aim of this study was to investigate whether there is allelic variation in *sdrD* gene among *S. aureus* isolates from healthy carriers in Tromsø 6 study and to determine if the *sdrD* variants differ in their adherence ability to selected human interaction partners.

DNA from 51 *S. aureus* isolates was extracted, and the A-region of *sdrD* was amplified, purified and the products were sequenced. The *sdrD* sequences were aligned, and the identities of the sequences were checked using BLAST.

To evaluate the contribution of *sdrD* variants in adhesion, a standard cloning method was used. A full-length *sdrD* variant was cloned into a shuttle vector and transformed into *E. coli* then into the gram-positive surrogate host *L. lactis.*

The finding from this study indicates that there is allelic variation in *sdrD* A-region among *S. aureus*. From a total of 51 clinical isolates; six different *sdrD* variants were revealed. Full-length SdrD1 variants was cloned into a shuttle vector and transformed into *E. coli.* The transformation of cloned SdrD1 into *L. lactis* failed and adhesion study could not accomplished.

Abbreviations

S. aureus ……*Staphylococcus aureus*

TSS ……...Toxic shock syndrome

CWG ……..…….Cell-Wall Glycopolymers

WTA .....Wall Teichoic Acid

LTA .....LipoTeichoic Acid

MSCRAMMs.....Microbial Surface Components Recognizing Adhesive Matrix Molecules

spa .....*S.aureus* Protein A

IsdA and IsdB .....Iron regulated Surface determinant protein A and B

SdrC, SdrD and SdrE .....Serine-aspartate repeat-encoding protein (C, D and E)

Cna.....Collagen binding protein

FnBPA and FnBPB.....Fibronectin binding protein A and B

CLfA and CLfB .....Clumping factor A and B

vWF .....von Willebrand factor

TNFRα.....Tumour-Necrosis Factor-α

Fg .....Fibrinogen

kDa .....kilo Dalton

ECM .....Extracellular matrix

ddH_2O.....double distilled water

HEK.....Human embryonic kidney cells

BLAST.....Basic Local Alignment Search Tool

1. Introduction

1.1 Staphylococcus aureus

Staphylococcus aureus (S. aureus) is a non-sporulation gram-positive bacterium, which belongs to a bacterial genus staphylococcaceae. It divides in several planes to form irregular clumps, and this form of arrangement helps to differentiate staphylococci from streptococci. On microscopic examination, this organism is 0.5 to 1.5 um in diameter. It occurs singly, in pairs, as short chains, or in grapelike clusters. *S. aureus* is typically hemolytic and forms pigmented yellow-orange colonies on blood agar. *S. aureus* is aerobic, catalase-positive, and oxidase negative organism. This bacterium is present in the normal microbial flora of the upper respiratory tract and the skin without causing any disease but at the same time it is a potential humans pathogen. (Todar, 2008-2012)

1.2 Colonization of *S. aureus*

S. aureus cells can survive for months on any type of surface (Crossley and Archer, 1997).

S. aureus colonizes the skin and mucosae of humans and several animal species. Multiple body sites can be colonized in humans, but the anterior nares of the nose are the most frequently colonized site by *S. aureus*. (Williams, 1963)

Humans carry *S. aureus* without symptoms that make prevention of *S. aureus* infections difficult. Studies show that about 20% of individuals are persistent nasal carriers, 30% are intermittent carriers, and about 50% non-carriers. (Kluytmans et al., 1997, Nouwen et al., 2004)

Individuals who carry *S. aureus* are in a higher risk for getting *S. aureus* infections and to transmit the bacteria to healthy individuals or patients. The risk of infection in intermittent carriers and non-carriers is similar. Patients often acquire *S. aureus* from healthcare personnel who are asymptomatic carriers. Carriers are often infected by their own strain carried in the nose. (White, 1963, Nouwen et al., 2005)

Adherence of *S. aureus* is one of critical steps for colonization. Colonization of *S. aureus* is multifactorial and determined by host and bacterial factors. (Weidenmaier et al., 2012) For example, individuals with HIV infection, intravenous drug users, diabetics, hemodialysis

patients, cystic fibrosis patients and hospitals inpatients have a high rates of colonization (Ten Broeke-Smits et al., 2010).

1.3 Pathogenesis of *S. aureus*

Ogston discovered Staphylococcal disease, the role of *S. aureus* in sepsis and abscess formation in 1880 (Ogston, 1882) however, *S. aureus* is still the second most common pathogen isolated and is remained to be a difficult and dangerous pathogen to treat in humans (Fehrmann et al., 2013).

S. aureus causes diseases such as acne, boils, phlebitis, impetigo, and many of these diseases cause the production of pus. It also causes life-threating pneumonia, osteomyelitis, endocarditis, meningitis, mastitis, urinary tract infections, arthritis, toxic shock syndrome (TSS) and food poisoning; (Todar, 2008-2012) resulting in considerable global human morbidity and mortality (Lowy, 1998).

S. aureus was isolated in 1880, and was sensitive to penicillin until 1946. While *S. aureus* developed resistance against penicillin, the new type of penicillin, Methicillin was arrived in 1959. It took only 2 years, before *S. aureus* was able to resist methicillin. Methicillin resistance *S. aureus* (MRSA) was identified in 1961 and responded only to very advanced antibiotics that were not meant to be the first-line treatment. Several studies have shown that the prevalence of MRSA varies geographically. The highest rates reported in Sri-lanka 86.5 % while in European countries especially in Netherland and Scandinavian the rate is less than 1%. (Stefani et al., 2012)

How can *S. aureus* overcome host defense?

S. aureus provides various strategies to escape from the host immune response. These strategies involve several types of enterotoxins and exotoxin that help to overcome a host's defense. In addition, *S. aureus* is able to resist certain cationic antimicrobial peptides by reducing the net negative charge of its cell wall and cell membrane. (Peschel, 2002)

Another method is that S. *aureus* neutralizes the killing mechanism of phagocytic cells by producing carotenoids and catalase (Van Belkum et al., 2002).

How *S. aureus* adhere to the host cell?

Colonization is one of the risk factor for infection. The key step for colonization is adherence. In order to colonize, *S. aureus* must adhere to the host cell and overcome host defense. (Johannessen et al., 2012, McCarthy and Lindsay, 2010, Vengadesan and Narayana, 2011)

S. aureus adheres to host cells by binding directly to epithelial cells, by invading sites of cellular damage, by migrating between endothelial cells and by bridging ligands (Lowy, 2000).

In a recent study, Triclosan that is used for over 40 years in personal care products and medical equipment's (soaps, toothpastes, catheters) to prevent infections was to promote nasal colonization of *S. aureus* (Syed et al., 2014). In another study, Staphylococcal H35A α-toxin was shown to inhibit adherence of *S. aureus (Yang et al., 2013)*. These studies are examples demonstrating how complicated the adherence of *S. aureus* to the host can be.

1.4 Cell-wall glycopolymer (CWG)

Gram-positive bacterial cell-wall contains peptidoglycan (Brown et al., 2013) and glycopolymers. Cell-wall glycopolymers (CWG) binds non-covalently to cell wall proteins. CWG are strain specific with variable structures and they have role in adhesion, virulence and assumed to prevent opsonization. CWG mutant *S. aureus* reduced biofilm formation. (Weidenmaier and Peschel, 2008)

Wall teichoic acid (WTA)

Wall teichoic acid (WTA) and lipoteichoic (LTA) acid are zwittrionic cell-wall polymer (Reichmann and Grundling, 2011).

WTA was discovered in 1950 and it is one of cell-wall glyco-polymers of gram-positive bacteria. It binds *S. aureus* to epithelial and endothelial cells and is assumed that it is essential for nasal colonization. (Weidenmaier et al., 2004)

WTA facilitates adhesions of *S. aureus* to the inner nasal cavity. Charged amino acids in WTA are responsible for this reaction. (Baur et al., 2014)

Studies showed that WTA mutant, that is lacks D-alanine is not capable of adhering to cotton rat nares (Weidenmaier and Peschel, 2008). WTA mutant bacteria were grow slower, sensitive to high temperature and unable to grow in high salt media. By altering bacterial cell surface, WTA play a role of protecting bacteria from antibiotics and host defense. (Brown et al., 2013)

Lipoteichoic acid (LTA)

LTA is bound to the bacterial membrane via glycolipid (Brown et al., 2013) and have a crucial part in cell division. It activates human Toll-like receptor TLR2 (receptor which recognizes pathogens). LTA is essential for the survival *S. aureus.* (Grundling and Schneewind, 2007)

LTA mutant *S. aureus* showed defects in biofilm formation and reduced ability to adhere (Fedtke et al., 2007).

1.5 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMS)

There are 22 proteins that are covalently attached to the cell wall of *S. aureus* by sortase A or B. Six proteins are ionic attached and one protein is attached trans-membrane (Mazmanian et al., 2002). Even though the mode of attachments is known, ligand specificity of the most of proteins has not been determined.

In addition, *S. aureus* adheres to host tissues by using its microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). MSCRAMMs recognize fibronectin, fibrinogen, collagen, and heparin related polysaccharides. They are responsible for the initial contact with host cells. (Patti et al., 1994)

MSCRAMMs are bound to the cell-wall peptidoglycan covalently, ionic and trans-membrane, but most of them are bounded covalently (Clarke and Foster, 2006).

MSCRAMMs are proteins that have adhesive properties such as, protein A (Spa), iron regulated surface determinant protein A (IsdA), serine-aspartate repeat-encoding protein (SdrC, SdrD and SdrE), collagen binding protein (Cna), Fibronectin binding protein A and B (FnBPA and FnBPB), clumping factor A and B (ClfA anf ClfB) and *S. aureus* surface protein G (SasG). (Foster and hook 1998; (Josefsson et al., 1998, Foster et al., 2014)

MSCRAMMs are a family of proteins that have the same structural organization (Figure 1) and a common mechanism for ligand binding. MSCRAMMs can bind to two or more ligands and are found in *Staphylococcus psedintermedius,* Coagulase negative Staphylococci, Entrococci and Streptococci. MSCRAMMs act as a virulence factor and are important in pathogenesis. (Foster et al., 2014)

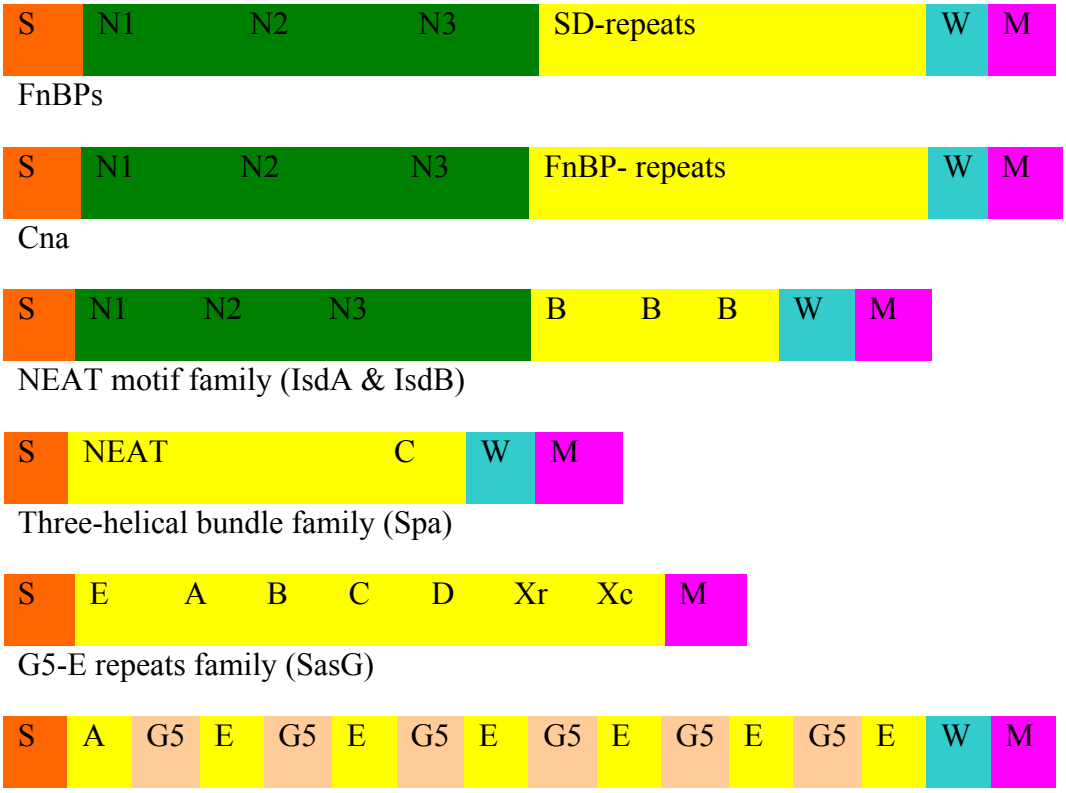

Figure 1: An image illustrates structural organization of MSCRAMMs. Clf-Sdr(Clumping factor Ser-Asp repeat protein), FnBPs (Fibronectin-binding proteins), Can (collagen binding protein), NEAT(Near iron transporter motif protein family(IsdA & IsdB), Spa (*S. aureus* protein A) SasG (*S. aureus* surface protein G). "S "represents signal sequences, "W" represents cell wall-spaning region and "M" sorting signal. Modified from (Foster et al., 2014).

1.5.1 S. aureus Protein A (Spa)

Spa is a surface protein of *S. aureus* which binds both von Willebrand factor (vWF) (Hartleib et al., 2000) and Tumour-Necrosis Factor-α (TNFRα) (Gomez et al., 2004). vWF are a large multimeric serum glycoprotein that mediates platelet adhesion at site of endothelial damage. TNFRα is a receptor for tumour-necrosis factor-α. In addition, Spa binds IgG molecules in a wrong orientation on their surface and that helps the bacterium avoid opsonization and phagocytosis. (Todar, 2008-2012)

Spa has a role in the pathogenesis of endovascular diseases by binding vWF (Hartleib et al., 2000).

1.5.2 The Ser-Asp dipeptide repeats (Sdr) family of proteins

S. aureus Sdr proteins have a potential adhesion function, which are involved in adherence to epithelial cells and contain putative B-domains (Lowy, 1998, Josefsson et al., 1998).

Sdr is derived from a repetition of amino acid serine –S- and aspartic acid –D. They are MSCRAMMs proteins involved in adherence to epithelial cells. (Corrigan et al., 2009)

The Sdr families in *S. aureus* are encoded *sdrC*, *sdrD* and *sdrE* genes (Josefsson et al., 1998), and the Sdr families in *S. aureus epidermids* are encoded *sdrG*, *sdrF* and *sdrH* (McCrea et al., 2000).

Sdr-families are structurally related to a family of cell wall anchored proteins known as ClfA and ClfB, with the exception of an additional B repeat domain of unknown function (Figure 2) (Josefsson et al., 1998).

SdrD has five tandem repeats (B5), while SdrC has two (B2) and SdrE has three (B3). The amino acid sequence identity of B2 with B5 is 97%; B2 with B3 and B3 with B5 is 94% similar. (Becherelli et al., 2013)

Signal sequence peptide (S), which is 75-90% similar among the sdr- families followed by a putative extracellular matrix (ECM) binding segment. A-region is identical with only 20-30% amino acid residues among the three members of the Sdr families. The A-region is followed by B-repeats. Ser-Asp residues dipeptides (R region) differentiate the Sdr families from ClfA

and ClfB. C-terminal to R-region is 83-97% identical among the Sdr families. (Josefsson et al., 1998)

1.5.2.1 Ser-Asp dipeptid repeats-D (SdrD) protein

SdrD is one of three-sdr family proteins, which plays a role in adherence to human nasal epithelial cell (Corrigan et al., 2009). The similarity of amino acids sequence identity between SdrC and SdrD is 34 %, and 43% between SdrD and SdrE (Becherelli et al., 2013).

The ribosome-binding sequences similarity of SdrD with SdrC and SdrE are 54% and 75%, respectively. In addition, 369 bp differentiates SdrD from SdrC, while 397 bp differentiates SdrD from SdrE. The other difference between SdrD and Sdr families is B-motifs. The A-region is divided into three sub-domains, which are named as N1, N2 and N3 (see Figure 2). There is 20-30% amino acid sequence similarity of the N domain between Sdr families. (Wang et al., 2013, Josefsson et al., 1998)

The expression level of SdrC, SdrD and SdrE during bacterial growth phase is different. SdrD expression is highest in early logarithmic growth phase, decreases in the mid logarithmic phase, and stays low until the stationary growth phase. Expression of SdrC and SdrE is similar. These are expressed in all growth phases and their expression declines in the stationary growth phase, but the difference between early logarithmic phase and stationary phase for SdrE is greater than for SdrC.(Sitkiewicz et al., 2011)

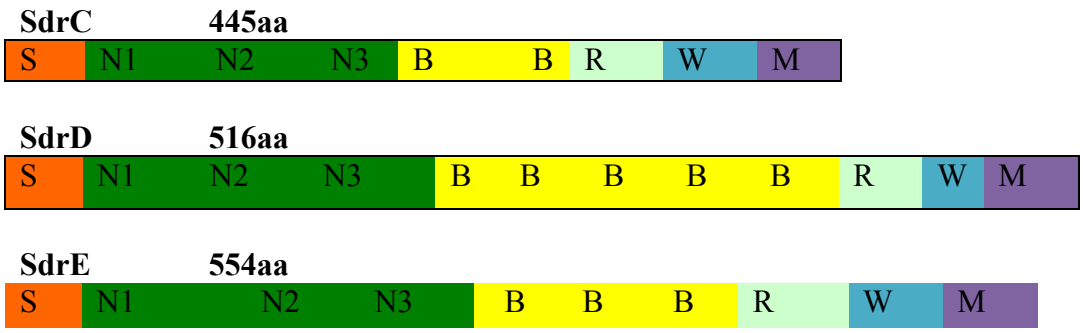

Figure 2: Structural organization of staphylococcal Sdr proteins families modified from(Josefsson et al., 1998) .S represents signal sequences, N1, N2, N3, represent putative ligand-binding in A region, B, represent B repeat, R represents the Ser-Asp dipeptide repeats, W represents cell wall-spaning segments and M represents membrane-spanning regions and positively charged residues**.** A-region of SdrD has 445 Amino acids, SdrD has 516 amino acids and sdrE has 554 amino acids.

1.5.2.2 The Fibronectin-binding proteins (FnbpA and FnbpB)

The Fibronectin-binding proteins are encodes by FnbA and FnbB. The two genes are closely linked but transcribed separately (Greene et al., 1995). The A-domain of FnbpA and FnbpB is 45% identical. It also shares 25% identity to the A-domain of ClfA and ClfB. The FnbpA and FnbpB A domains bind to the C terminus of the γ-chain of fibrinogen at the same site as ClfA and the platelet integrin. (Wann et al., 2000)

Fibronectin-binding proteins FnbpA and FnbpB bind to fibrinogen and elastin as well as to Fibronectin (Roche et al., 2004). FnBp binds to the host protein Fibronectin that bridges the bacteria to host receptor named integrins on the cell surface (Figure 3)(Foster, 2002). Elastin is a hydrophobic protein that provides resilience and elasticity to tissues such as lung, aorta, and skin. Tropoelastin is a soluble precursor of elastin, which is produced by smooth muscle cells, endothelial cells, chondrocytes and fibroblasts. (Roche et al., 2004)

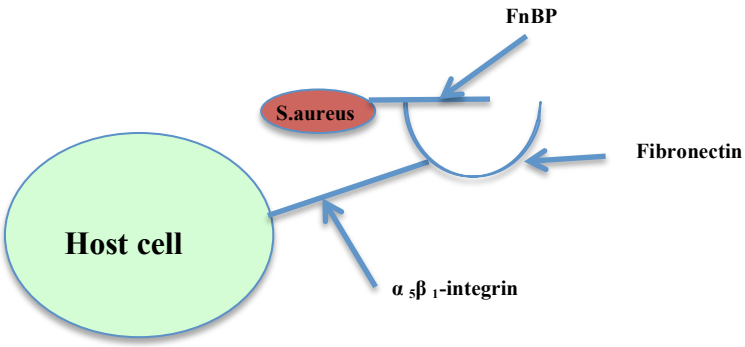

Figure 3: The contribution of FnBP for bacterial attachments to host cells (adapted from (Foster, 2002))

1.5.2.3 Clumping factor A and B (ClfA and Clf B)

ClfA and ClfB are members of a family of proteins that are covalently anchored to the cell wall peptidoglycan. ClfA of *S. aureus* binds to the Fibrinogen (Fg) γ-chain in the blood. ClfB binds to the α-chain of fibrinogen as well as to cytokeratin 10. The key difference between ClfA and ClfB is that ClfA binds to the C terminus of the Fg γ-chain, whereas ClfB binds to the Fg α-chain at a site that is flanked by many residues on either side of the binding region. (Ponnuraj et al., 2003)

The other difference of ClfA and ClfB is that they are present in bacteria in different stages of growth phases. ClfA is present in all stages of growth, while ClfB is found in early exponential phase and is absent in stationary phase. (Foster and Hook, 1998)

ClfA subdomains N2N3 (Figure 1) are promote fibrinogen binding. The subdomain N1 is required for ClfA to be expressed and localized to the *S. aureus* cell wall. (McCormack et al., 2014)

Fg is a 340-kDa glycoprotein that is found at a concentration of around 9 μM in the blood. It is composed of six polypeptide chains, two α-, two β-, and two γ-chains, which are arranged in a symmetrical di-meric structure. Fg is an important clotting protein that participates in controlling blood loss following vascular injury. (Herrick et al., 1999)

2. Aim of the study

Increasing bacterial resistance to almost all available antibiotics causes an urgent need for new antimicrobial drugs, drug targets and therapeutic concepts (Høiby, 2000). In order to obtain future targets for prevention and therapy, it is important to increase the understanding of the molecular aspects of interaction between *S. aureus* and the host cells.

Bacterial surface proteins are important to the success of bacterial strains survival (Papa et al., 2013) therefore they are novel candidates for the development of new antimicrobial drugs or vaccines. The combined vaccines with IsdA, IsdB, SdrD and SdrE have produced partial protection against *S. aureus in vivo* animal model, but failed in clinical trial (Stranger-Jones et al., 2006).

The aim of this study is to evaluate whether there is allelic variation in *sdrD* gene among *S. aureus* isolated from healthy carriers in Tromsø 6 study and to determine if the *sdrD* variants differ in their adherence ability to host cells.

To determine the contribution of *sdrD* variants in adhesion, a standard cloning method will be used, where full-length *sdrD* variants will be cloned into the shuttle vector and transformed into the gram-positive surrogate host *Lactococcus lactis (L. lactis)*. Thereafter adhesions study will be done under the same condition in order to determine adherence ability.

3. Materials

3.1 Bacterial isolates

A total of 51 *S. aureus* isolates were included and an overview of isolates described in Appendix 1.

The bacterial isolates used in this study were from Tromsø 6 study. The Tromsø 6 study was the multipurpose population based study and carried out from 2007-2008. The study included 12,984 men and women aged 30-87. A total of 4026 men and women aged 30-49 years had a nasal swab taken. (Olsen, 2013)

Among the *S. aureus* isolates from the Tromsø 6 study, 554 were scanned for *sdrD* and 28.88 % were positive while 71.2 % were negative (unpublished results from research group). We simply took the first 51 on the list who were *sdrD* positive. The number 51 is easy to manage for our study.

E. coli strain DH5 and *L. lactis* strain M1363 competent cells were used for preparing competent cells in this study.

3.1.2 *sdrD* reference strains

S. aureus NCTC8325 and *S. aureus* MSSA476 were used as control strains for optimization of PCR. The *sdrD* DNA sequences and amino acid sequences from *S. aureus* strains NCTC8325 (Acc.nr.NC_007795.1), MSSA476 (Acc.nr.NC_002953.3), N315 (Acc.nr.NC_002745.2), Ho50960412 (Acc.nr.NC-017763.1), MW2 (Acc.nr.NC_003923.1) and Newman (Acc.nr.NC_009641.1) were used as reference sequences in alignment. Reference sequences were obtained from GenBank.

3.2 Plasmid

Two different pMG36e plasmids were used; one pMG36e plasmid containing SdrD from *S. aureus* strain NCTC8325 (pMG36e:SdrD_NCTC8325) and one "empty plasmid" pMG36e without sdrD. These were kindly provided by Professor Dzung Bao Diep Norwegian University of life sciences, Ås (NMBU) (Figure 4 and Figure 5).

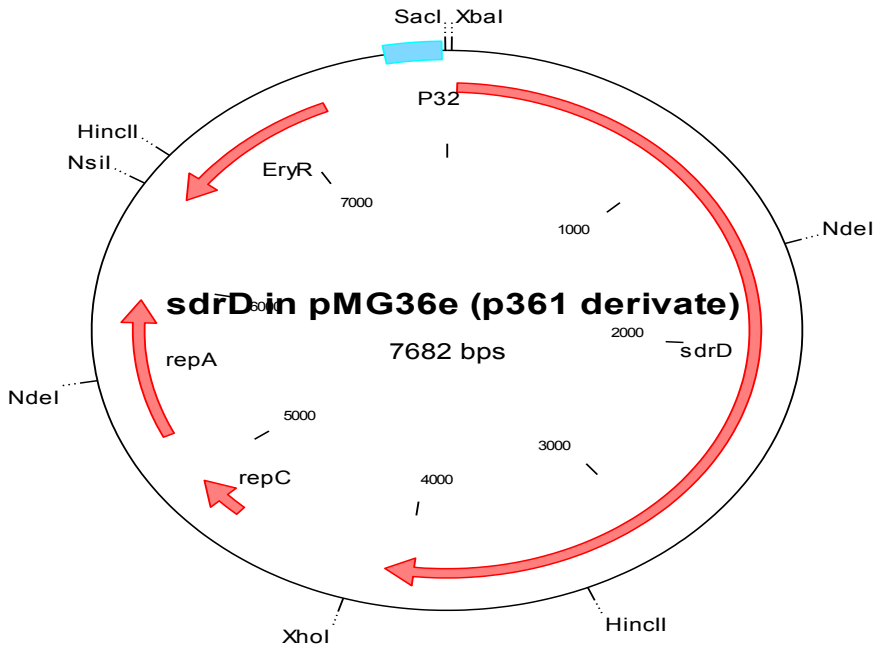

Figure 4: Cloning vector pMG36e containing SdrD from *S. aureus* strain NCTC8325 (pMG36e:sdrD_NCTC8325) (professor DB. Diep, 2012)

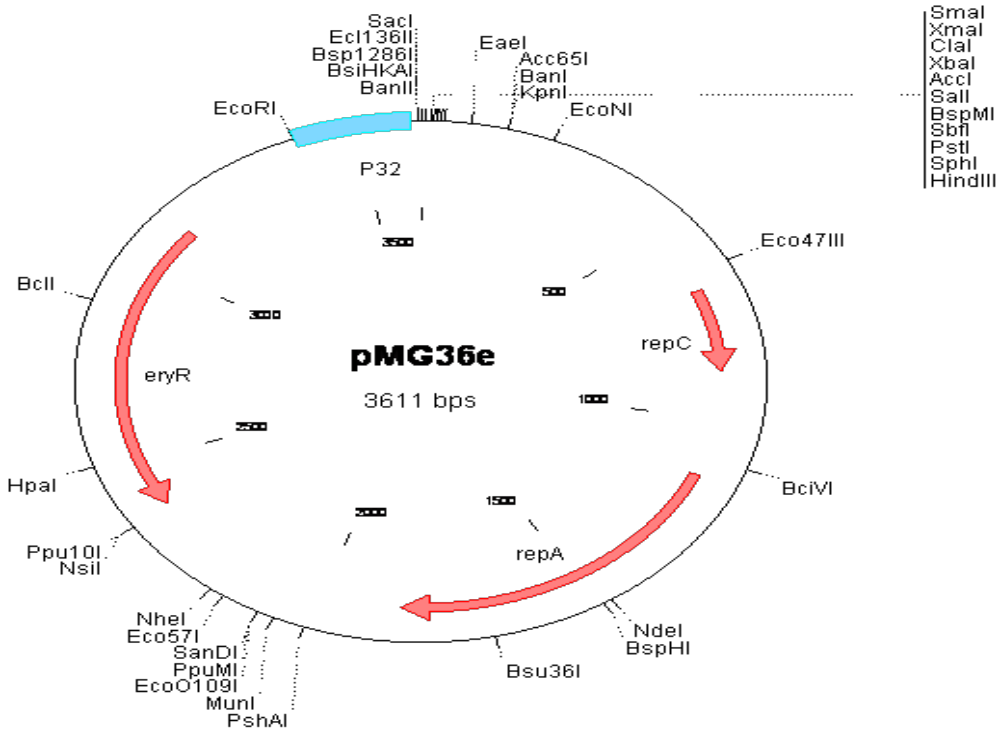

Figure 5: Cloning vector Empty pMG36e. pMG36e vector without sdrD (professor DB. Diep, 2012)

3.3 Primers, materials, chemicals and reagents

Table 1: List of primers

	Manufacturer	Sequence (5'-3')	Reference
SdrD-A-F	Sigma	GGAACCAAGAAGCAAAGGCTG	(Xue et al., 2011)
SdrD-A-R	Sigma	CTTCTTGACCAGCTCCGCCAC	(Xue et al., 2011)
SdrD-forw	Sigma	AGTTGATGACAAAGTTAAATCAGGT	This study
SdrD-rev	Sigma	TAATATCTTCCGGATTCAATCCA	This study
amh100F	Sigma	AAAAGAGCTCTGAATTAGGAGTAATCTAATGCT	This study
amh203R	Sigma	ATCAGCATGCGATACATTTCATAAATTTTGAAATA	This study
PMg36e-Forw01	Sigma	TATTCGGAGGAATTTTGAAATGGC	This study
SdrD-rev-2	Sigma	TGCAGTATCATGTTTTGCAGTCG	This study

Primers were obtained freeze-dried and the stock solutions had a concentration of 100µM. The working solution was diluted to a concentration of 10µM.

Table 2: Primer used and amplification length

Primer		Used for PCR and Sequencing	Length
Forward	Reverse		
SdrD-A-F	SdrD-A-R	SdrD-lang	1540 bp
SdrD-A-F	SdrD-rev	SdrD-700	765 bp
SdrD-forw	SdrD-A-R	Sdrd-800	840 bp
amh100F	amh203R	Full-length SdrD	3447 bp
PMg36e-Forw01	SdrD-rev-2	Sequencing	

S	A-REGION (N1, N2, N3)	B1	B2	B3	B4	B5

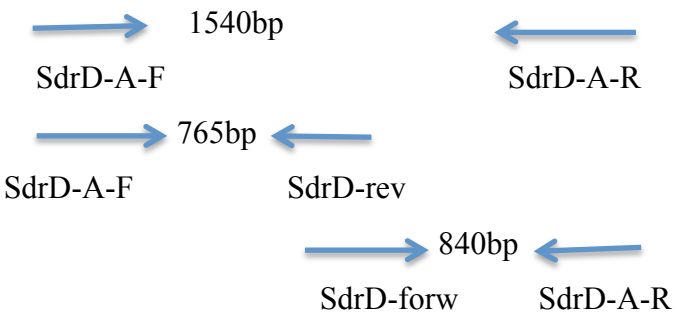

Figure 6: Primers map. sdrD-A-F and sdrD-A-R used for sdrD-lang PCR, sdrD-A-F and sdrD-rev used for sdrD-700 PCR, sdrD-forw and sdrD-A-R used for sdrD-800 PCR

Table 3: List of chemicals and reagents

Chemicals	Catalog number	Manufacturer
Reddy-mix-PCR master mix, 1x	AB-0575/DC/LD	Thermo scientific
25mM $MgCl_2$	R0971	Thermo Scientific
TE-Buffer 1x pH:8 (See Appendix 3 for composition)	Tris-base, T-5941 EDTA, E-5134	Sigma
TAE-buffer 1x (See Appendix 3 for composition)	Tris-base, T-5941 EDTA, E-5134	Sigma
Seakem Le agarose	50004	Lonza Rockland ME
Gel Red nucleic acid stain	41003-1	Biozium
1 kb plus DNA ladder	10787-026	Invitrogen
Big dye v.3.1 (Ready mix from sequence analyze lab)	1304236	Life technology
5X sequencing buffer (Ready mix from sequence analyze lab)	1103132	Life technology
ExoSap-It (10 U/µl)	78200	Affymetrix.USA
Phusion DNA polymerase (2U/µl)	0181109	Biolabs
dNTP(10mM)	D7295	Sigma
5X phusion GC buffer	0011309	Biolabs
DreamTaqGreen PCR master mix (2X)	00156088	Thermo Scientific
BSA 100x, working solution BSA (10X)	0181109	Biolabs

Buffer1.1 (1x)	0011306	Biolabs
SacI (10 U/µl)	0501305	Biolabs
SphI (10 U/µl)	0481304	Biolabs
XbaI (10 U/µl)	R7260	Sigma, Germany
XhoI (10 U/µl)	R0146s	Sigma, Germany
NEBuffer. 4 (1x)	0011309	Sigma, Germany
SGM17-Gly medium	103K0183	Sigma, Germany
Electrotransformation solution	57H0144	Sigma, Germany
Washing Solution		Sigma, Germany
M17 medium	103K0183	Oxoid, England
Blood agar plate		
SGM17 agar plate	SM17 + 0.5%glucose	Sigma, Germany
SGM17 medium with 10µg/ml erythromycin	See composition in Appendix 3	
SOC medium	See composition in Appendix 3	
LB broth medium with 400µg/ml erythromycin	See composition in Appendix 3	

Table 4: List of instruments and materials

Instruments	Name	Manufacturer
Centrifuge	Biofuge Pico	Heraeus
	Avanti J-26 XP	Beckman Coulter, USA
	F-5430R	Eppendorf AG, Germany
Nanodrop spectrophotometer	ND-100	Saveen Werner
PCR machine	T100 GeneAmp 9700	Bio-Rad, Singapore Applied biosystem, Singapore
GFX PCR DNA and Gel Band Purification Kit	28-9034-71	GE-healthcare, UK
G:Box	F3	Syngene Cambridge, UK
Vortexer	119448347	Heidolph, Germany
NucleoBond xtra midi column kit	11091008	MN, Germany
NucleoBond xtra mini column kit	1112006	MN, Germany
MicroPulser™ Electroporator	165-2100	Bio-Rad, USA

4. Methods

DNA from a total of 51 *S. aureus* strains was isolated, and the A-region of *sdrD* was amplified by PCR, purified and the products were sequenced. The identity of the sequence was checked using Basic Local Alignment Search Tool (BLAST) program. All sdrD sequences were aligned using the program BioEdit version 7.2.5.

Strain nr 32 was selected as representative for sdrD1 for insert preparation. Genomic DNA was isolated; full length PCR was performed, the PCR product checked on 1 % agarose gel, the product purified, restriction enzyme digestion was performed, and the product was visualized again on 1% agarose gel for restriction enzyme specification and used for ligation. Vector was isolated; restriction enzyme digestion was performed, the product was checked on 1 % agarose gel, purified, and used for ligation.

4.1 DNA Sequencing

4.1.1 Rapid chromosomal DNA isolation by boiling

1. Bacteria were picked from a frozen pure culture by scratching the sterile loop across the surface of the culture.
2. The loop was used to streak out the cells on the surface of a blood agar plate.
3. The plate was incubated at 37 °C overnight.
4. 8-10 bacteria colonies were dissolved in 200µl TE-buffer pH=8 in an eppendorf tube.
5. The sample was centrifuged at 5000rpm for 5 minutes.
6. The supernatant was discarded and the cell pellets were re-suspended in 100µl TE-buffer
7. pH = 8.
8. Incubated for 10 minutes at 100 °C.
9. The tubes were quickly chilled on ice and centrifuged at 5000 rpm for 5 minutes.
10. The supernatant was transferred to another eppendorf tube and stored at -20 °C.

4.1.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method used to make millions of identical copies (amplification) of a piece of DNA *in vitro*. Double-stranded DNA to be amplified is heated to about 94-98 °C for 30x cycles. DNA is denatured by high temperature to separate individual strands. The temperature is lowered to about 40-65 °C or 2-10 °C lower than the melting temperature for the primers, so that primers can react and stick to the short DNA pieces, which are complementary to the sequence of the strand to be amplified and not to each other. Then the temperature is raised to 72 °C and the polymerase replicates the DNA from 3' -OH end of the primers. Then two new DNA strands are gained from one starting DNA strand. The entire new DNA created acts as template for new DNA, so that the increase will be exponential. (Sambrook et al., 1989)
Gradient PCR includes the use of gradually decreasing annealing temperature in cycling reaction to find the optimal annealing temperature for primer pair combination(Padmakumar and Varadarajan, 2003).

Preparation of sample for PCR

1. Concentrations in the final working PCR mixture were 0.5x Reddy-mix, 0.5µM forward and revers primers (see Table 2 for primer list) and 5µl or 3µl templates. The volume was adjusted to 25µl by double distilled water.
2. The PCR mixture was mixed well and PCR run. (Table 5)

Table 5: Cycling program used to amplify DNA from *S. aureus sdrD* A-region for strain nr.1 to nr.51

SdrD-700 and SdrD-800		
Procedure	Temperature	Duration
Initial denaturation	94 ˚C	2 min
Repeat cycling	30X	
Final denaturation	94 ˚C	30 sec
Annealing	60.4 ˚C	30 sec
Initial elongation	72 ˚C	50 sec
Final elongation	72 ˚C	3 min
Cooling	4 ˚C	∞

Optimization of PCR

An optimized PCR amplification will produce a single, bright band on gel. To get a single, bright band on a gel one has to optimize the enzyme concentration, magnesium concentration, denaturation temperature and time, the annealing temperature and time, and cycles. Very high enzyme concentration and too many cycles may give nonspecific background products. Incomplete denaturation, very low enzyme concentration and low cycles reduce desired product yield. While very high or long denaturation leads to loss of enzyme activity. The magnesium concentration may affect primer annealing, strand dissociation temperature of both template and PCR product and specificity. (White, 1990)

4.1.3 Determination of DNA concentration

Due to the aromatic bases within its structure, DNA absorbs UV light at 260 nm. Purines (thymine, cytosine and uracil) and pyrimidines (adenine and guanine) both have peak absorbance at 260 nm, thus making NanoDrop spectrophotometer suitable for quantifying DNA samples. NanoDrop spectrophotometers can also be used to quantify proteins using direct absorbance. (Desjardins and Conklin, 2010)

Proteins and phenolic compounds have strong absorbance at 280nm, because of aromatic amino acid side chains (tryptophan, phenylalanine, tyrosine and histidine) within proteins. Similarly, the aromatic of phenol groups of organic compounds absorbs strongly near 280 nm. (Desjardins and Conklin, 2010)

Pure nucleic acids yield 260/280 ratio of around 1.8 for DNA, and a 260/280 ratio of around 2.0 for RNA. Acidic blank solutions sample will under-represent the ratio by 0.2-0.3, while a basic blank solution sample will over-represent the ratio by 0.2-0.3. Significantly different purity ratio may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. (Desjardins and Conklin, 2010)

Procedure

1. The upper and lower optical surfaces of the NanoDrop spectrophotometer sample retention system were cleaned by pipetting 5µl of clean deionized water on to the lower optical surface.
2. The lever arm was closed to ensure that the upper pedestal comes in contact with the deionized water, and both optical surfaces were wiped off with a clean, dry, lint-free lab wipe.
3. The NanoDrop software was opened and the Nucleic Acid application was selected. Dispensing 1.5µl of deionized water onto the lower optical surface performed a blank measurement.
4. Once the blank measurement was completed, both optical surfaces were cleaned with a clean, dry, lint-free lab wipe.
5. Dispensed 1.0µl of DNA sample onto the lower optical pedestal and closed the lever arm.
6. Selected “Measure” in the application software. And the software automatically calculated the DNA concentration.

4.1.4 Gel electrophoresis

Gel electrophoresis is a method for separating and identifying proteins or fragments of DNA or RNA in a gel made of agarose or polyacrylamide between thin glass plates. The method can detect bands that contain 1-10ng DNA. Agarose gels have lower resolving, but greater range of separation than polyacrylamide. The two ends of the gel in contact with each electrode buffer. Negatively charged molecules at the appropriate pH will migrate towards the anode (positive pole). The walking speed depends on the size of the molecules to be separated, voltage across the gel, pore size and concentration of the gel material. One often uses a dye bromphenolblue to see how far the electrophoresis has reached. (Sambrook et al., 1989)

Preparation of 1% agarose Gel

1. 1 g Seakem® agarose was weighed and mixed in 100 ml TAE-buffer solution to make 1% gel.
2. The mixture was heated to boil temperature in a microwave oven, until when the solution was dissolved and clear
3. The agarose was allowed to cool slightly, mixed with 5µl gelred and poured into the casting tray.
4. Thereafter the agarose gel was left to solidify for 25 minutes.

Agarose gel electrophorese

1. The gel in its casting tray was placed in a buffer chamber connected to a power supply.
2. Running buffer (TAE 1x) was poured into the chamber until the gel was completely covered.
3. The comb was withdrawn to form the wells into which PCR sample will be loaded.
4. When all wells have been loaded with sample, the power supply switched on with 100V, 200mA.
5. Electrophoresis for 1 hour and 15 minutes, the gel was then moved and photographed using G: BoxF3.

4.1.5 Purification of PCR-Product

Before starting the sequencing reaction, it is important to remove excess dNTP, DNA polymerase, and primer dimer from the PCR-products. ExoSapIt was used to purify the PCR-product. ExoSapIt contain two hydrolytic enzymes, Exonuclease I and shrimp alkaline Phosphatase. Exonuclease I degrades residual single stranded primers and single strand DNA. Shrimp alkaline phosphatase hydrolyzes the remaining unwanted dNTPs, which interferes with sequencing from the PCR mixture. (Hanke and Wink, 1994, Dugan et al., 2002, Kim and Blackshaw, 2001)

Purification of PCR- product using ExoSapIt

1. 0.7U Exo-SAPIt was added to each 15µl of PCR-product and the tubes were placed in the PCR thermo cycler.
2. The Cycling-program was adjusted (Table 6).
3. The purified PCR-products were stored at 4°C while waiting for sequencing.

Table 6: Cycling program for ExosapIt

Procedure	Temperature	Duration
Degrades & Hydrolyzes	37 °C	1 min
	37 °C	60 min
Inactivate ExoSapIt	85 °C	15 min
Cooling	4 °C	∞

4.1.6 Sequencing of DNA

DNA sequencing is the method used to characterize and confirm order of nucleotide in DNA. The method used to characterize the identity of newly cloned DNA, confirm the identity of a clone and PCR products. It is also used to identify polymorphism. (Sambrook et al., 1989, Hanke and Wink, 1994)

The enzymatic method developed by Frederick Sanger and coworkers in 1977. Because of its comparative ease, the Sanger method was soon automated and was the method used in the first generation of DNA sequencing. The methods can directly sequence only relatively short 300-1000 nucleotide long DNA fragments in a single reaction. (Sambrook et al., 1989)

In this study, A 3130 Genetic Analyzer (ABI/Life Technologies) and BigDye Terminator v.3.1 cycle-sequence reaction reagent was used to perform direct sequencing. DNA sequence was analyzed by capillary electrophoresis automatically and the result was presented as an electro-photogram.

Preparation of sample for sequencing

1. PCR-products purified with ExoSap- It
2. The final concentration in the PCR mixture was 0.75x sequencing buffer, 0.5µM primer forward or revers (see Table 2 primer list), 3.0µl template (purified by EXoSapIt), 1.0µl v.3.1Bigdye and 12.0µl ddH_2O.
3. The PCR mixture mixed well and PCR was run at program in Table 7.
4. The samples were delivered to the sequencing laboratory to analyze by capillary electrophoresis (A 3130 Genetic Analyzer).

Table 7: Sequencing cycling program

PCR program		
Procedure	Temperature	Duration
Initial denaturation	96 ˚C	1 min
Repeat cycling	30 ×	
Final denaturation	96 ˚C	10 sec
Annealing	50 ˚C	5 sec
Initial elongation	60 ˚C	4 min
Final elongation	4 ˚C	15 min
Cooling	4 ˚C	∞

4.2 Bioinformatics

BLAST is a program for aligning query sequences against those present in a selected target database. The program is provided by National Center for Biotechnology Information (NCBI) and access point for this tool is http://blast.ncbi.nlm.nih.gov/Blast.cgi. (Johnson et al., 2008)

BioEdit is a biological sequence alignment editor program. The program is used to manipulate and analyses of sequences. The program offer the tools required to create and manipulate restriction enzyme mapping, annotatable plasmid drawing, and several built in analysis options. (Hall, 1999)

The phylogenetic tree was obtained from http://www.phylogeny.fr. This is free online data program that can used to estimate the evolution of a genetically related group of organisms and identification of homologous sequences. The program can analyze both DNA sequence and amino acids sequence. (Dereeper et al., 2008)

One click mode is used to create the phylogenetic tree (see figure 19 & 20). One click mode is designed for biologist with no experience in bioinformatics to create phylogenetic tree. The

set of DNA sequence or amino acid sequence submitted (copy, paste) in FASTA format and after a few minutes the phylogenetic tree displayed. (Dereeper et al., 2008)

4.3 Cloning

Cloning involves several basic molecular biology techniques, such as PCR, DNA agarose gel electrophoresis, enzymatic reaction, ligation and bacterial transformation. Cloning is using nuclear DNA from one organism to create a second organism with the same nuclear DNA. Sub-cloning is cutting a piece of DNA from one organism and inserting it into a plasmid where it can be replicated by a host organism. (Griffiths et al., 1999)
There are two major DNA components; a plasmid and an insert that are to be joined into a single DNA molecule. The key steps in sub-cloning are preparing insert DNA, Preparing plasmid, cutting insert and plasmid with the same enzyme at precise location, joining two DNA fragments covalently, transformation to a host cell, selecting host cells that containing recombinant DNA. (Griffiths et al., 1999)
Insert and plasmid are generated by digestion with appropriate restriction enzymes, and the complementary ends of the plasmid and insert are then joined by DNA ligase (Griffiths et al., 1999).

4.3.1 Preparation of full length *sdrD* Insert

One *S. aureus* strain (strain nr. 32) was selected as representative for SdrD1. By using lysis method genomic DNA was isolated from *S. aureus*. Full-length *sdrD* was amplified by PCR (Table 8). PCR product was checked on 1% agarose gel and purified by using GFX PCR DNA and Gel band purification kit. Purified DNA was cut by restriction enzyme.

Isolation of genomic DNA from *S. aureus* by lysis method

1. Bacteria were picked from a frozen pure culture by scratching the sterile loop across the surface of the culture.
2. The loop was used to streak out the cells on the surface of a blood agar plate.
3. The plate was incubated at 37 °C overnight.
4. A small loop of bacteria from overnight plate re-suspend in 100µl lysismix and incubated for 30 min at 37 °C

5. The sample was spun down at 4500 rpm for 5min; the supernatant was transferred in a new tube and placed at 100 °C for 5 min.
6. 200µl TE-buffer pH-8 (1X) was added and stored at -20 °C

Preparation of master mix for PCR

1. Master mix was prepared for each 20µl reaction by mixing, 1x Phusion GC buffer, 0.5µM forward and reverse primers (See Table 2 for primer list), 0.2mM dNTP, 0.02U Phusion DNA polymerase, 1.5µl template and the final volume adjusted by adding double distilled water.
2. PCR was run as explained in table 8.

Table 8: Full length SdrD amplifying PCR cycling Program

PCR Program		
Procedure	Temperature	Duration
Initial denaturation	98 °C	30 sec
Repeat cycling	30x	
Final denaturation	98 °C	10 sec
Annealing	61.1 °C	15 sec
Initial elongation	72 °C	3 min
Final elongation	72 °C	10 min
Cooling	4 °C	∞

Extraction and purification of DNA from agarose gel

GFX PCR DNA and Gel band purification kit was used to purify full-length *S. aureus sdrD* PCR-product from Gel band. GFX MicroSpin column involves four main steps, capturing of DNA, binding, washing and drying, and eluating. In capture step, protein will be denatured and agarose gel dissolved, then binding buffer promotes the binding of DNA to a glass fiber

matrix. Washing buffer washed away proteins and salt contamination. At the last step purified DNA is eluted in a low ionic strength buffer. (Marko et al., 1982)

Procedure

1. 1.5 ml micro centrifuge DNase-free tube was weighed and the weight was recorded. Using a clean scalpel, the agarose band containing the sample of interest was cut out. The agarose gel band was placed in a pre-weighted DNase-free 1.5 ml micro centrifuge tube. The weight of the micro centrifuge tube containing the agarose band was recorded and the weight of the agarose slice was calculated.
2. 10µl capture buffer type 3 was added to for each 10 mg of gel slice. The mixture was incubated at 60 °C for 20 minutes until the agarose was completely dissolved. The mixture was inverted every 3 minutes.
3. If the color of solution was yellow, there was no need to adjust pH. If not see the protocol to adjust the pH.
4. The sample mix was loaded onto the assembled GFX MicroSpin column and collection tube, and spun at 13000 rpm for 30 seconds.
5. The flow was discarded and the GFX MicroSpin column was placed back inside the collection tube.
6. From wash buffer type-1, 500µl was added to GFX MicroSpin column, and spun at 13000 rpm for 30 seconds.
7. The flow was discarded and the GFX MicroSpin column was transferred to a new micro centrifuge collection tube.
8. 25µl Elution buffer type 4 was added to the center of the membrane in the assembled GFX MicroSpin column, and spun at 13000 for 1 minute to recover the purified DNA.

Restriction enzyme digestion for full-length *sdrD* insert

1. Master mix was prepared by mixing 9.2ng template (purified), 0.1x Buffer1.1, 1x BSA, 0.2U SacI and SphI, and 13µl ddH_2O.
2. The sample was incubated at 37 °C for 4 hour.
3. The product was checked on 1 % agarose gel for restriction enzyme specification.

4.3.2 Preparation of plasmid

Two different pMG36e plasmids were used; Alternative one pMG36e plasmid containing sdrD from *S. aureus* strain NCTC8325 (pMG36e:SdrD_NCTC8325) and alternative 2 "empty" pMG36e without sdrD. Both plasmids were isolated by the same procedure from *L. lactis*.

Plasmid isolation using NucleoBond xtra midi/mini column kit

Plasmids are small, circular, extra-chromosomal DNA molecules found in bacteria cells. Plasmids carry genes for antibiotic resistance and replicate independently of the bacteria genome. (Griffiths et al., 1999)
The basic principles are lysis of bacterial cells by an optimized set of formulated buffers based on Sodium hydroxide (NaOH) or Sodium dodecyl sulfate (SDS) (Birnboim and Doly, 1979).
When the bacterial cell pellet are treat with buffer RES and lysed by LYS buffer, proteins, chromosomal and plasmid DNA are denatured and RNA is degraded. Then neutralization buffer NEU precipitate SDS by pulling down proteins. Chromosomal DNA, and plasmid DNA can revert to its native structure and remains in solution. Then equilibration buffer EQU and washing buffer WASH is used to remove all traces of contaminants. Elution is carried out by neutralize the anion exchange resin and plasmid DNA is released. (Macherey, 2012)

Procedure

1. *L. lactis* colony with PMG36e plasmid (both type plasmid) was cultivated in 100 ml SGM17 media with 10µg/ml erythromycin and was incubated at 32 °C overnight without shaking.
2. After overnight incubation the whole contents of flask was transferred to a plastic bottle with lid.
3. The sample was centrifuged at 6000 rpm for 20 minutes.
4. The supernatant was discarded and the pellet was re-suspended in 4ml PBS buffer, which contained 1mg/ml lysozyme (5µl of 200 mg/ml).
5. The sample was incubated at 37 °C for 2 hours in water bath.
6. 4ml RES buffer was added to the sample and mixed well.
7. 4ml Lysis buffer was added and shaken gently. Thereafter incubated for 5 minutes in room temperature.
8. NucleoBond xtra midi column kit was prepared as mentioned in protocol by washing with 12 ml EQU buffer.
9. After 5 minutes of incubation 8ml NEU buffer was added to the sample and shaken slowly to mix.
10. The whole content of the sample was transferred to the Column and 5ml EQU buffer was added to the column.
11. The filter of column was removed and 8ml WASH buffer was added directly to the sample.
12. 5 ml ELU buffer was added to the column.
13. The column was removed and 4 ml Isoprpanol was added to the sample.
14. The sample was centrifuged at 15000 rpm for 30 minutes.
15. The supernatant was discarded and 2 ml 70% ethanol was added to the pellet. (NB! Difficult to see the pellet)
16. The sample was centrifuged at 15000 rpm for 10 minutes and the supernatant discarded.
17. The pellet was dried for 1 hour in room temperature.
18. The pellet was dissolved in 200µl TE (1X) buffer and was transferred to Eppendorf tube.
19. The concentration was determined by nano-drop spectrometry and the sample was stored at -20 °C.

Isolation of plasmid from *E. coli using* NucleoBond xtra midi column kit

1. Colonies was inoculated in 100 ml LB medium with erythromycin 400µg/ml and incubated at 37 °C overnight while shaking at 225rpm.
2. After overnight incubation the whole contents of flask was transferred to a plastic bottle with lid
3. The sample was centrifuged at 6000 rpm for 20 minutes.
4. 8ml RES buffer was added to the sample and mixed well.
5. 8ml Lysis buffer was added and shaken gently and incubated for 5 minutes in room temperature.
6. NucleoBond xtra midi column kit was prepared as mentioned in protocol by washing with 12 ml EQU buffer.
7. After 5 minutes incubation 8ml NEU buffer was added to the sample and shaken slowly to mix.
8. The whole content of the sample was transferred to the Column and 5ml EQU buffer was added.
9. The filter of column was removed and 8ml WASH buffer was added directly to the sample.
10. 5ml ELU buffer was added to the column.
11. The column was removed and 4 ml Isoprpanol was added to the sample.
12. The sample was centrifuged at 15000 rpm for 30 minutes.
13. The supernatant was discarded and 2 ml of 70% ethanol was added to the pellet. (NB! Difficult to see the pellet)
14. The sample was centrifuged at 15000 rpm for 10 minutes and the supernatant was discarded.
15. The pellet was dried for 1 hour in room temperature.
16. After 1 hour the pellet was dissolved in 200µl TE (1X) buffer and transferred to an eppendorf tube.
17. The concentration was determined by nano-drop spectrometry and stored at -20 °C.

Restriction enzyme digest for plasmid pMG36e:SdrDNCTC8325

1. 16.1ng template, 0.1x Buffer 4, 1x BSA, 0.2 U XbaI and XhoI and 33µl ddH_2O, were mixed to prepare the sample.
2. The sample was incubated at 37 °C for 4 hour.
3. The product was checked on 1% agarose gel.

Restriction enzyme digest for plasmid pMG36e from *L. lactis* or *E. coli*

1. 18.7ng template, 0.1x Buffer1.1, 1x BSA, 0.2U SacI, and SphI and 33µl ddH_2O, were mixed to prepare the sample.
2. The sample was incubated at 37 °C for 4 hour.
3. The product was checked on 1% agarose gel.

4.3.3 Ligation

Ligation is the process of joining two pieces of DNA from different sources together with the formation of covalent bonds. DNA ligase is the enzyme used to catalyze ligation reaction and requires ATP. (Pascal, 2008)

T4 DNA ligase catalyzes both blunt end and cohesive end ligation. To prevent both type of ligation, it needs to adjust ATP concentration. Increasing ATP concentration to 5mM gives inhibition of the blunted end ligation, and further increasing of ATP concentration to 7.5mM inhibited both ligations. (Ferretti and Sgaramella, 1981)

Preparation of ligation mix

1. Master mix was prepared with three different vector:insert ratio (Table 9)
2. The sample was incubated at 16 °C overnight.
3. After overnight incubation, the sample transformed into *E. coli.*

Table 9: Ligation mix with three different pMG36e(plasmid): strain nr.32(insert) ratio

Ligation mix per tube					
1:1		1:3		1:10	
16µl	ddH_2O	13µl	ddH_2O	6µl	ddH_2O
2µl	T4 buffer	2µl	T4 buffer	2µl	T4 buffer
0.5µl	pMG36e	1µl	pMG36e	1µl	pMG36e
0.5µl	Strain nr.32	3µl	Strain nr.32	10µl	Strain nr.32
1µl	T4 Ligase	1µl	T4 Ligase	1µl	T4 Ligase

Transformation of plasmid DNA (pMG36e) into *E. coli*

1. The competent *E. coli* (DH5) bacteria cells and isolated plasmid DNA (PMG36e) was melted on ice.
2. 200µl competent bacteria cells were added to each ice-cold falcon tube.
3. 2µl plasmid DNA (from overnight incubated ligation mix) was added to one of cold falcon tube.
4. Incubated on ice for 30 minutes.
5. The cells were heat shocked at 42 °C (water bath) for 90 seconds.
6. The sample was placed on ice for 90 seconds.
7. 800 µl S.O.C added to each Falcon tube.
8. Incubated at 37 °C while shaking at 225 rpm for 45 minutes.
9. 200 µl of the solution plated out on LA^+-plates with erythromycin 400µg/ml.
10. Incubated at 37 °C overnight.

4.3.4 Colony screening and selection of transformants

1. 10.5µl ddH_2O, 1x DreamTaq Green, 0.4µM forward and revers primers (see Table 2 primer list) and 1 colony (from incubated plate one colony was picked with a sterile toothpick) were mixed to prepare the sample.
2. The same toothpick was used to streak on new LA^+-plates with erythromycin 400µg/ml for further use and incubated at 37 °C overnight.

3. Cycling program was adjusted (Table 10).
4. PCR-products were checked on 1% agarose gel.

Table 10: Insert screening PCR

Cycling Program		
Procedure	Temperature	Duration
Initial denaturation	94 °C	5 min
Repeat cycling	35x	
Final denaturation	94 °C	30 sec
Annealing	55 °C	10 sec
Initial elongation	72 °C	2 min
Final elongation	72 °C	7 min
Cooling	4 °C	∞

Plasmid isolation from *E. coli* using NucleoBond xtra mini column kit

1. Colonies that contained the right insert was inoculated in 5 ml LB medium with erythromycin 400µg/ml and incubated at 37 °C overnight while shaking at 225rpm.
2. The sample was centrifuged at 11000 x g for 30 seconds at room temperature and supernatant was discarded.
3. 250µl buffer A1 was added to the sample and the pellet was re-suspended by pipetting up and down.
4. 250µl buffer A2 was added to the sample and incubated for 5 minutes after mixing by inverting the tube.
5. 300µl buffer A3 was added to the sample and mixed by inverting the tube.
6. The sample was centrifuged at 11000 x g for 10 minutes at room temperature.
7. The supernatant was transferred to Nucleospin plasmid column (max. of 750µl) and centrifuged for 1 minute at 11000 x g. This step was repeated to load the remaining sample.

8. Preheated (to 50 °C), 500µl buffer AW was added to the sample and centrifuged for 1 minute at 11000 x g.
9. The supernatant was discarded and centrifuged for 2 minutes at 11000 x g and the collection tube was discarded.
10. The column was placed on a new collection tube and 50µl buffer AE added.
11. The sample was incubated for 1 minute at room temperature and was centrifuged for 1 minute at 11000 x g

4.3.5 Identification of *E. coli* transformants containing pMG36e:SdrD1

1. To identify the right constructs (pMG36e:SdrD1) sequencing was done.
2. 12µl ddH_2O, 0.75x sequencing buffer, 0.5µM PMG36e-Forw01 or 0.5µM SdrD-rev-02 (see Table 2 primer list), 1.0µl of V.3.1 Bigdye and 6ng template was mixed to prepare the sample.
3. Construct was confirmed by DNA sequencing (see Table 7 for sequencing cycling program)

4.4 Electro-transformation

4.4.1 Competent cells

Competent cells are cells that are exposed to change the state of the cell wall; this implies change in permeability of the cell membrane, to get the ability of a cell to take up extracellular DNA. Competence of cells occurs under natural, chemical and electronic condition. Natural competence occurs when bacteria express specialized proteins that allow the cell to bind and take up large pieces of DNA from environment. This could be advantageous due to its role in horizontal gene transformation. Under chemical competence, the cells are incubated in a solution under cold condition and exposed to heat shock, while electro competence cells are briefly shocked with an electric field. (Mercenier and Chassy, 1988)

Procedure

1. 100µl *L. lactis* strain M1363 cells was plated out on the surface of SGM17-Gly plate and incubated over night at 30 °C.
2. 5ml of SGM17-Gly was inoculated with the overnight colonies and incubated overnight at 30 °C
3. 100ml of SGM17-Gly was inoculated with 100µl of the overnight culture, the cells were grown at 30 ˚C to an OD_{600} of 0.5 - 0.8
4. The culture was transferred into two pre-chilled 50 ml falcon tubes.
5. The sample was centrifuged at 4500 rpm for 15 min at 4 °C and the supernatant were discarded.
6. The pellet was re-suspended with 50 ml ice-cold washing solution by pipetting up and down, and vortex for 1 minute
7. The sample was spun down at 4500 rpm at 4 °C for 15 min, the supernatant was discarded, the tubes were inversed to remove all traces of washing solution
8. The sample was washed again with 50ml washing solution, spun down at 4500 rpm at 4 °C for 15 minutes, supernatant was discarded and the tubes were inversed to remove all traces of washing solution
9. The pellets were re-suspended again with 10 ml of ice-cold washing solution and the sample was transferred in a new 50 ml tube, the same procedure was repeated 2 times.
10. The sample was spun down at 4500 rpm for 15 min at 4 °C, supernatant was discarded, tubes were inversed to remove all traces of washing solution
11. The pellet was re-Suspended in 1 ml ES solution, aliquot 100 µl in sterile 1.5 ml tubes, and store at -70 °C

4.4.2 Electro-transformation

Electro-transformation also known as electroporation is the most efficient tool for plasmid DNA uptake. It involves the application of high-voltage electric field pulses of short duration to induce the formation of transient pores in the membranes of cells. (McIntyre and Harlander, 1989)

Electro-transformation efficiency is depending on number of cell washes to electroporation, cell number, DNA amount and cell growth phase. For high degree of success cell of high resistance and very low conductivity is required. The basic protocol involves preparation of cells, which usually are harvested in mid-log phase, following a series of washing steps, and applied electric field pulse. After application of the pulse, the cells are diluted in ice-cold medium and plated on the appropriate selective medium. (Gasson and vos, 1994)

Electro-transformation of Constructs (pMG36e:sdrD1) from *E. coli* into *L. lactis*

1. A tube (100µl) of electro-competent *L. lactis* strain M1363 cells and purified pMG36e:sdrD1 DNA from *E. coli* was melted on ice.
2. 2µl of plasmid DNA was added to 100µl of electro-competent *L. lactis* strain M1363 cells on ice and incubated for 5 minutes
3. The sample was transferred to a 0.2 cm electro-poration cuvette
4. The sample was electroporated with 2,5 kV/25µF/400 Ω
5. 900µl of SMG17-Gly was added immediately and homogenized by pipetting up and down
6. The cells suspension was transferred to a sterile 1.5ml tube and incubated for 3 hours in a water bath at 30 °C
7. 100µl the cells suspension was plated out on SMG17 with 10µg/ml erythromycin agar plate and incubated at 30°C for 48 hours
8. The colonies were spread out to a new SMG17-ery 10µg/ml agar plate for further use.

Plasmid isolation from *L. lactis* NucleoBond using xtra mini column kit

1. A small loop of bacteria colonies was re-suspended in 5ml SMG17-ery 10µg/ml media and incubated overnight at 30 °C
2. The sample was centrifuged at 4500 rpm for 15 minutes
3. The pellet was re-suspended in 125µl PBS buffer containing 1mg/ml lysozyme and incubated for 2 hours on a water bath at 37 °C
4. 250µl A1 buffer was added to the sample and mixed well by inverting the tube.
5. 250µl A2 buffer was added and mixed gently and incubated for 5 minutes in room temperature.
6. 300µl A3 buffer was added to the sample and mixed well by inverting the tube.
7. The sample was centrifuged for 10 minute at 11000xg at room temperature.
8. The supernatant was transferred to the Nucleospin plasmid Column and centrifuged for 1 minute at 11000x g and supernatant was discarded.
9. 500µl AW buffer was heated at 50 °C and added directly to the sample, thereafter centrifuged for 1 minute at 11000xg and supernatant was discarded.
10. 600µl A4 buffer was added to the column, centrifuged for 1 minute at 11000xg and supernatant was discarded.
11. Centrifuged again for 2 minutes at 11000xg and the collection tube was discarded.
12. The column was transferred to a new tube; 50µl AE buffer was added, incubated for 1 minute at room temperature and centrifuged for 1 minute at 11000xg.

The transformants were checked for the presence of construct (pMG36e:sdrD1) by insert screening (see 4.3.4 for procedure**).** Insert screening sample mix was modified; 2.0µl plasmid DNA template was used instead of 1 colony.

5. Results

5.1 Isolation of DNA from *S. aureus*

To evaluate allelic variation among *sdrD* from *S. aureus* isolates, it was needed to isolate DNA before doing PCR and sequencing. DNA from *S. aureus* strains MSSA476 and NCTC8325 were isolated. The purity and concentration of the DNA isolated from strain MSSA476 and NCTC8325 was measured by Nanodrop spectrometry. The absorbance was read at 260nm.

The results showed that the purity of nucleic acids yield (260/280 ratio) was 1.88 for strain MSSA476 with concentration 99.46 ng/µl. 1.79 for strain NCTC8325 with concentration 24.46 ng/µl. The purity 1.88 and 1.79 were suggested that the DNA should be pure enough to proceed. The DNA purity and concentration of the 51 *S. aureus* strains was not determined.

5.2 Optimization of *sdrD* A-region PCR

SdrD-lang PCR

For detecting the full-length A-region of *sdrD* (SdrD-lang, 1540 bp), the PCR program was needed to optimize. Two bacterial strains *S. aureus* strain MSSA476 and *S. aureus* strain NCTC 8325 were used for optimization. Isolation of DNA for both strains was performed as described under "method", and purity of DNA was measured with NanoDrop spectrophotometer.

SdrD-lang PCR was run using the program given in Table 11. As the Figure 7 shows, SdrD-lang *S. aureus* strain NCTC8325, lane "B" gave one single bright band at size approximately 1540 bp, but SdrD-lang *S. aureus* strain MSSA476 lane "A" gave single band at size around 1540 bp and additional weak multiple bands.

Table 11: sdrD-lang cycling program

sdrD-Lang		
Procedure	Temperature	Duration
Initial denaturation	95°C	5 min
Repeat cycle	30×	
Denaturation	95°C	30 sec
Annealing	62°C	30 sec
Initial elongation	72°C	160 sec
Final elongation	72°C	4 min
Cooling sample	4°C	∞

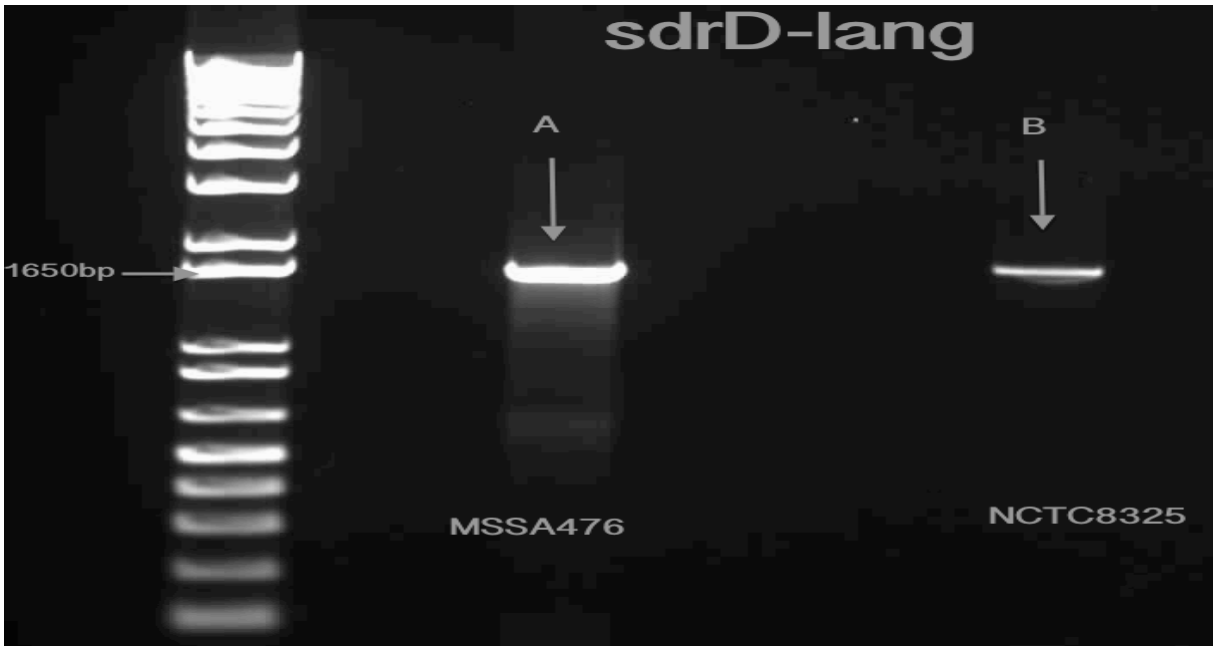

Figure 7: sdrD-lang on 1% agarose gel; molecular weight standard (1kb plus), lane A *S. aureus* strain MSSA476, and lane B *S. aureus* strain NCTC8325.

Optimization of sdrD-lang PCR

Since the result of SdrD-lang PCR for *S. aureus* strain MSSA476 gave additional multiple weak bands, the optimization of PCR was necessary.

This was done by optimized elongation time (initial elongation time in Table 11, 160 sec changed to 140 sec), and the result showed one single bright band at approximately 1540 bp for sdrD-lang *S. aureus* strain MSSA476 as expected (Figure 8).

The sdrD-lang PCR for both *S. aureus* strains (MSSA476 and NCTC8325) was optimized (Figure 8), but the size was not suitable for sequence analysis at the sequencing unit at the department. Because of the limitation of the read length of the DNA sequencer (the read length is only up to 1000bp sequences), the sdrD A-region sequence was divided into two parts, 765bp (SdrD-700) and 840bp (SdrD-800).

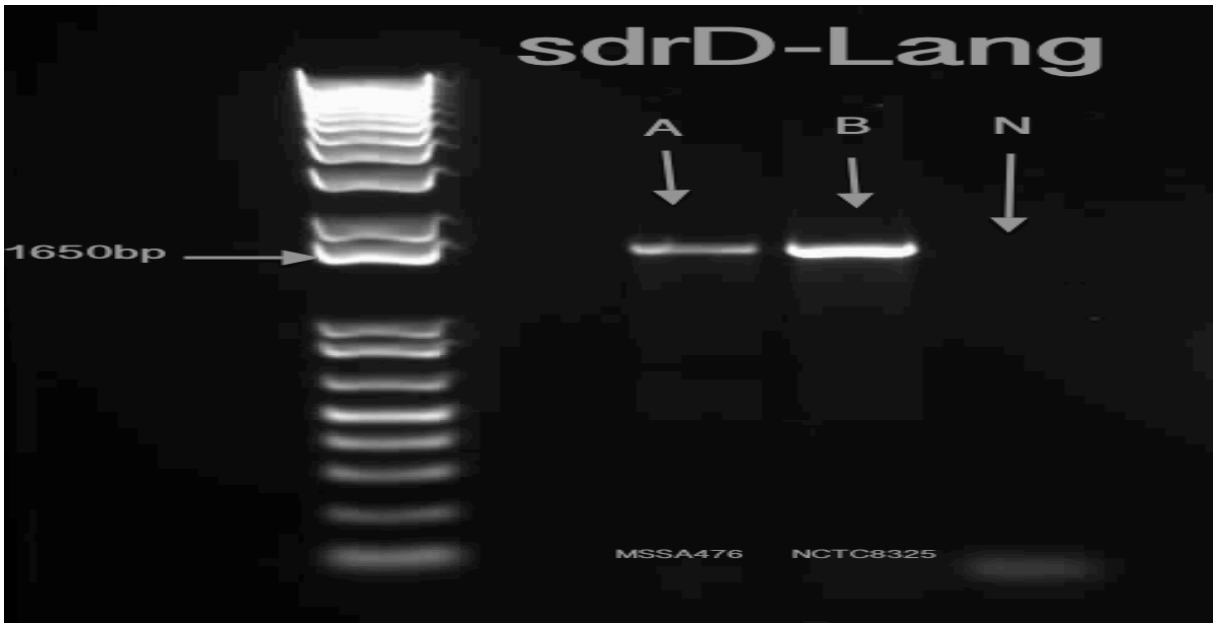

Figure 8: An image of SdrD-lang on 1 % agarose gel; molecular weight standard (1Kb plus), lane A MSSA476-lang, lane B NCTC8325-lang and lane N negative control

SdrD-700 and sdrD-800

The initial PCR was performed using the cycling program given in Table 12. The result from sdrD-700 and sdrD-800 showed a fragment of 850 bp in addition to several weak multiple bands for both *S. aureus* strains (strain MSSA476 and strain NCTC8325) (see Figure 9). A

single bright band was expected at a size 765 bp for sdrD-700 and 840 bp for sdrD-800. Therefore optimization continued by changing $MgCl_2$ concentration.

Table 12 Cycling program for sdrD-700 and sdrD-800 for strains MSSA476 AND NCTC8325

sdrD-700 and sdrD-800		
Procedure	Temperature	Duration
Initial denaturation	94 ˚C	2 min
Repeat cycling	30×	
Final denaturation	94 ˚C	30 sec
Annealing	60 ˚C	30 sec
Initial elongation	72 ˚C	50 sec
Final elongation	72 ˚C	2 min
Cooling	4 ˚C	∞

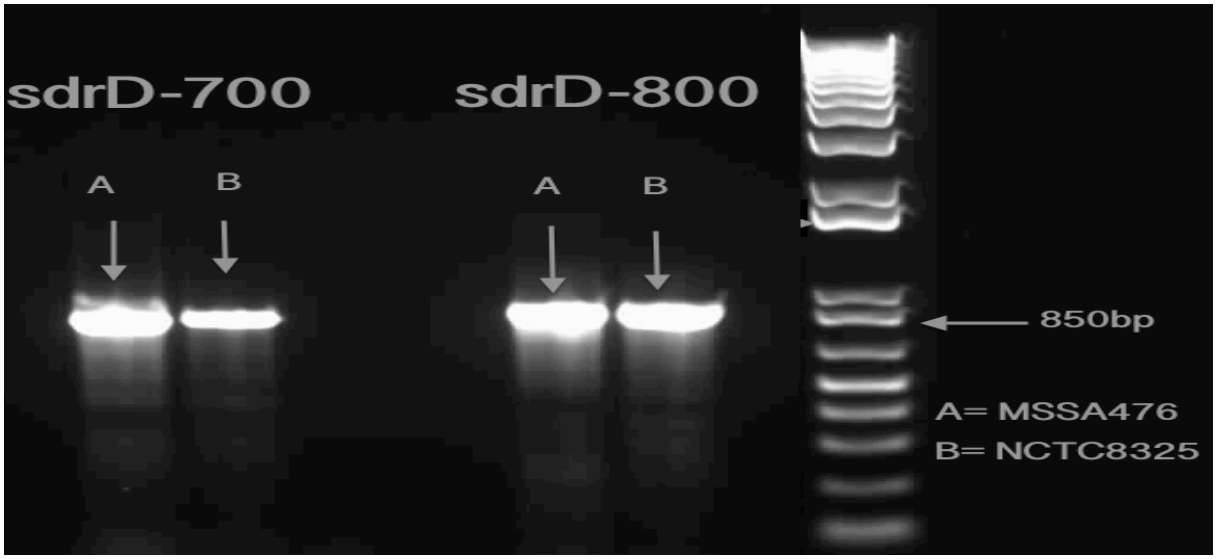

Figure 9: 1% agarose gel. Molecular weight standard (1Kb plus), lane A PCR product from strain MSSA476 sdrD-700 (left) and sdrD-800 (right), lane "B" PCR product from strain NCTC8325 sdrD-700 (left) and sdrD-800 (right).

Optimization by changing $MgCl_2$ concentration for sdrD-700 and sdrD-800 PCR

The PCR was run using the cycling program shown in Table 12.Three different $MgCl_2$ concentrations were tested, 2.5mM, 2.0mM and 1.5$MgCl_2$.

The sdrD-700 PCR with $MgCl_2$ concentration of 2.5mM resulted in one single bright band at a size 850bp, but produced additional weak multiple bands with 2.0mM and 1.5mM for strain MSSA476. (Figure 10)

The sdrD-800 PCR with $MgCl_2$ concentration of 1.5mM resulted one single bright band at a size 850bp, but produced additional weak multiple bands with 2.5mM and 2.0mM for strain MSSA476 (see Figure 10).

The sdrD-700 PCR with all $MgCl_2$ concentration resulted one single band and extra weak multiple bands at 850bp for strain NCTC8325. (Figure 10)

The sdrD-800 PCR with $MgCl_2$ concentration of 2.5mM and 1.5mM resulted one single bright band at a size 850bp, but 2.0mM produced extra weak multiple bands in addition to single band for strain NCTC8325 (see Figure 10).

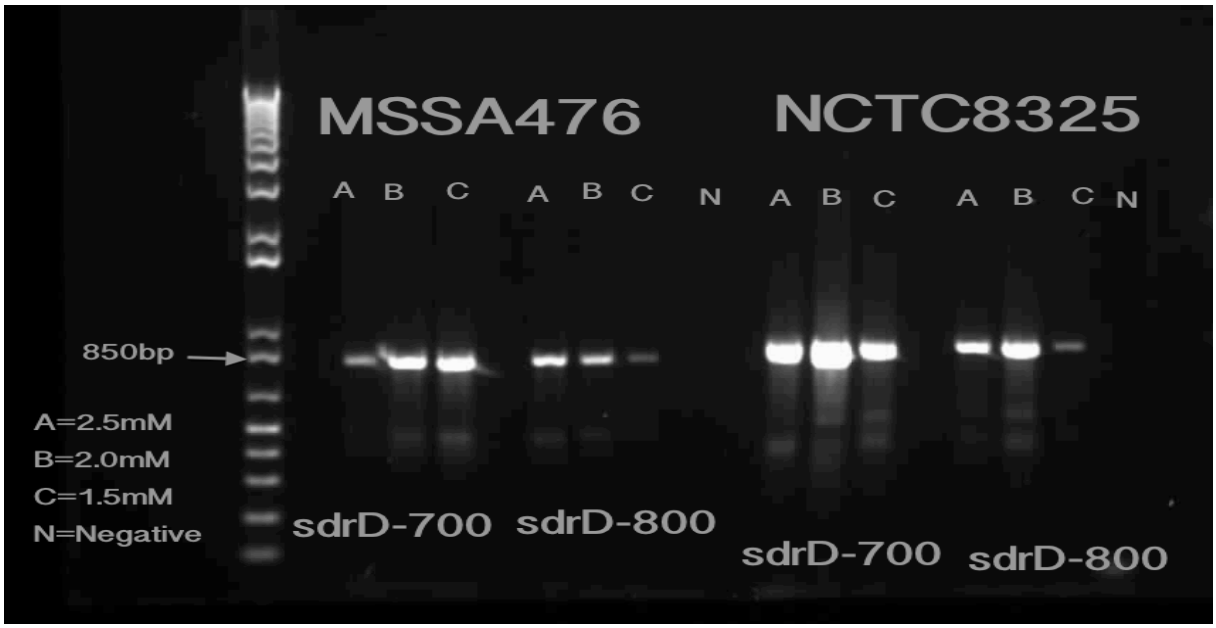

Figure 10: An image of 1% agarose gel and PCR cycling program that illustrate an optimization of PCR by different concentration of MgCl2. molecular weight standard (1Kb plus), sample in lane A, contain 2.5mM $MgCl_2$, lane B, Contain 2.0mM $MgCl_2$, lane C 1.5mM $MgCl_2$.

Optimization of annealing temperature by gradient PCR

Since the results were yet inconclusive, gradient PCR was used to optimize the annealing temperature (See Table 13) so as to get one bright single band for both (SdrD-700 and SdrD-800).

From a total of 1.5mMol $MgCl_2$ concentration obtained one bright single band at 850 bp at 60.4 °C (lane C) for SdrD-700 and SdrD-800 PCR for *S. aureus* strain NCTC8325 (see Figure 11).

For *S. aureus* strain MSSA476 one bright single band at 850 bp was achieved at 62.0 °C (lane B) for SdrD-700 PCR (see Figure 12).

For SdrD-800 PCR one weak single bright band at a size 850 bp observed at 63.0 °C (lane A), 62.0 °C (lane B) and at 50.0 °C (lane H) (see Figure 12).

PCR product from annealing temperature that gave single bright band was sequenced. PCR product from strain NCTC8325 gave a good sequence result, but from strain MSSA476 no sequence result was obtained.

Since there was an unexpected single band at 50.0 °C (lane H, figure 12) and no sequence result were obtained from the strain MSSA476 DNA, *S. aureus* strain NCTC8325 was chosen for further use as positive control.

The final PCR program that used to amplify *S. aureus* strain 1-51 is shown in Table 5.

Table 13: Cycling program sdrD-700 and sdrD-800 for strains MSSA476 AND NCTC8325

sdrD-700 and sdrD-800 PCR		
Procedure	Temperature	Duration
Initial denaturation	94 ˚C	2 min
Repeat cycling	30x	
Final denaturation	94 ˚C	30 sec
Annealing	63-50 ˚C	30sec
Initial elongation	72 ˚C	50sec
Final elongation	72 ˚C	2 min
Cooling	4 ˚C	∞

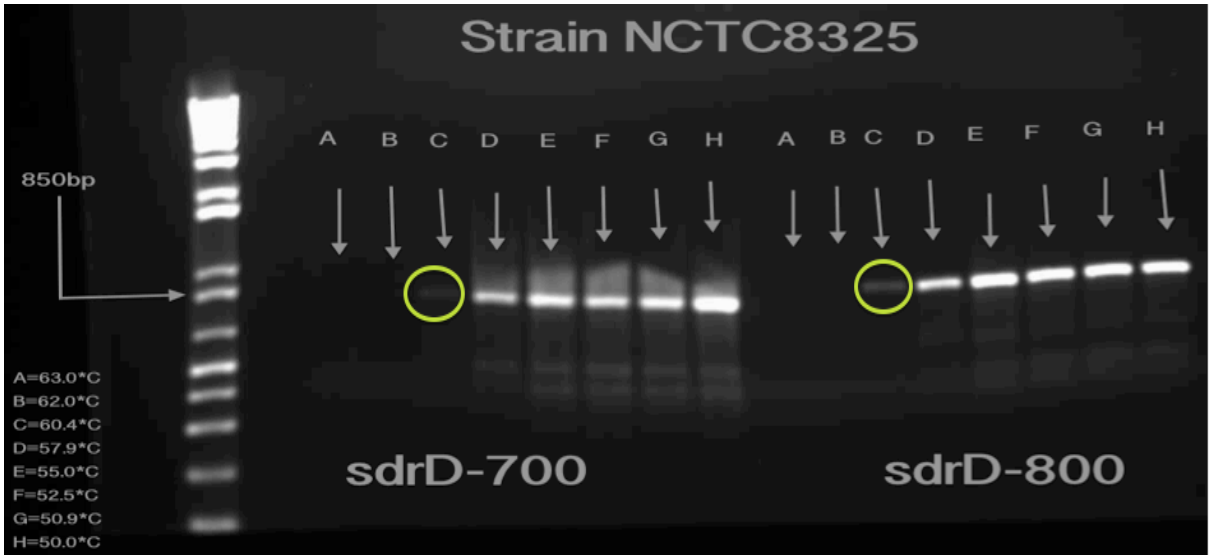

Figure 11: An image of 1% agarose gel showing the result of optimization by annealing temperature *S. aureus* strain NCTC8325, molecular weight standard (1Kb plus), highest temperature in lane A (63 °C) and lowest temperature in lane H (50 °C). The green circle around lane C shows the best results.

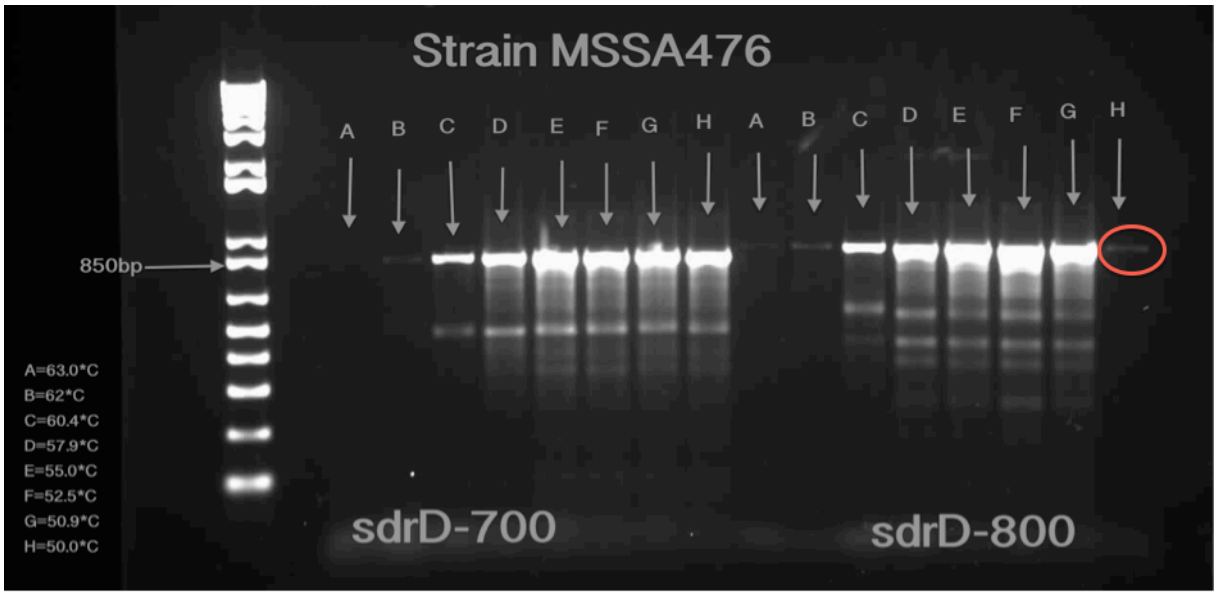

Figure 12: An image of 1% agarose gel showing the result of optimization by annealing temperature *S. aureus* strain MSSA476, molecular weight standard (1Kb plus), highest temperature in lane A (63 °C) and lowest temperature in lane H (50 °C). The red circle around lane H shows an expected band.

5.3 SdrD-700 and SdrD-800 PCR for *S. aureus* strains nr.1 to 51

PCR program for strain NCTC8325 was optimized as indicated in Table 5. This program was used to run sdrD-700 and sdrD-800 PCR on 51 *S. aureus* strains that isolated from healthy carriers in Tromsø 6 study.

SdrD-700 PCR product

As it is shown in Figure 13, for strain nr.1 to 20, one single bright band at a size 850 bp was obtained only from DNA strain nr.2 at optimal PCR program. Multiple weak bands for sdrD PCR product from strains nr.5 and nr.13 and strong multiple bands for the remaining strains was obtained in addition to single band.

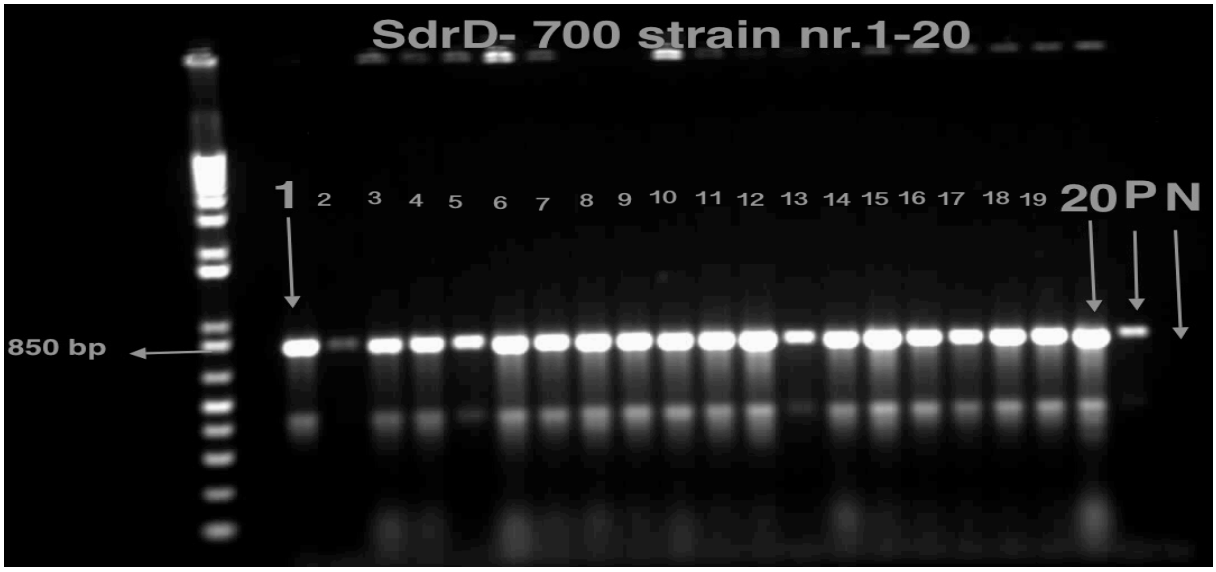

Figure 13: An image of 1% agarose gel showing the result of SdrD-700 PCR for *S. aureus* strain nr.1 to 20(from left to right), molecular weight standard (1Kb plus), in Lane "1" strain nr.1, lane "2" strain nr.2 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive control and Lane "N" negative sample that contain water instead of DNA template.

SdrD-700 PCR product from strains nr.21 to 51 gave one single band. This is illustrated in Figure 14 and figure 15. The size of band was around 850 bp. The expected size of SdrD-700 PCR product was approximately 765 bp.

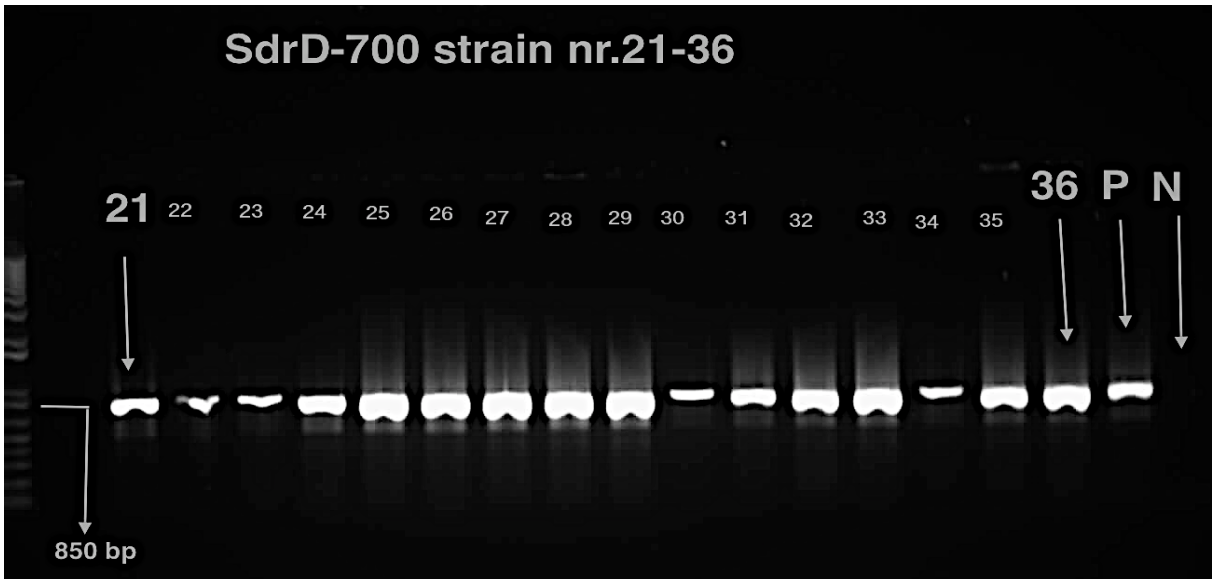

Figure 14: An image of 1% agarose gel showing the result of SdrD-700 PCR for *S. aureus* strain nr.21 to 36(from left to right), molecular weight standard (1Kb plus), in Lane "21" strain nr.21.lane "22" strain nr.22 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive control and Lane "N" negative sample that contain water instead of DNA template.

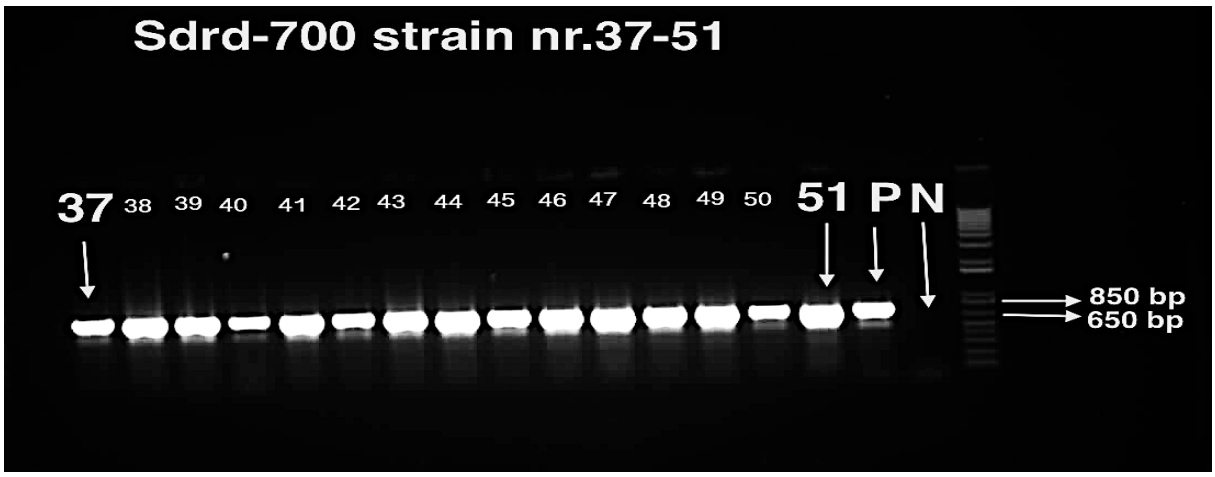

Figure 15: An image of 1% agarose gel showing the result of SdrD-700 PCR for *S. aureus* strain nr.37 to 51(from left to right), molecular weight standard (1Kb plus), in Lane "37" strain nr.37, lane "38 " strain nr.38 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive control and Lane "N" negative sample that contain water instead of DNA template.

SdrD-800 PCR product

As it shown in Figure 16 sdrD-800 PCR product from strain nr.1 to 20, strain nr.2 (lane 2) did not gave a product and positive control (lane P) gave a very weak band at optimal PCR program. SdrD-800 PCR product from strain nr 3, 5,9,13 and 16 gave one single band at a size 850bp and rest of the strains gave additional weak multiple bands. While one single band at a size 840 bp was the expected band.

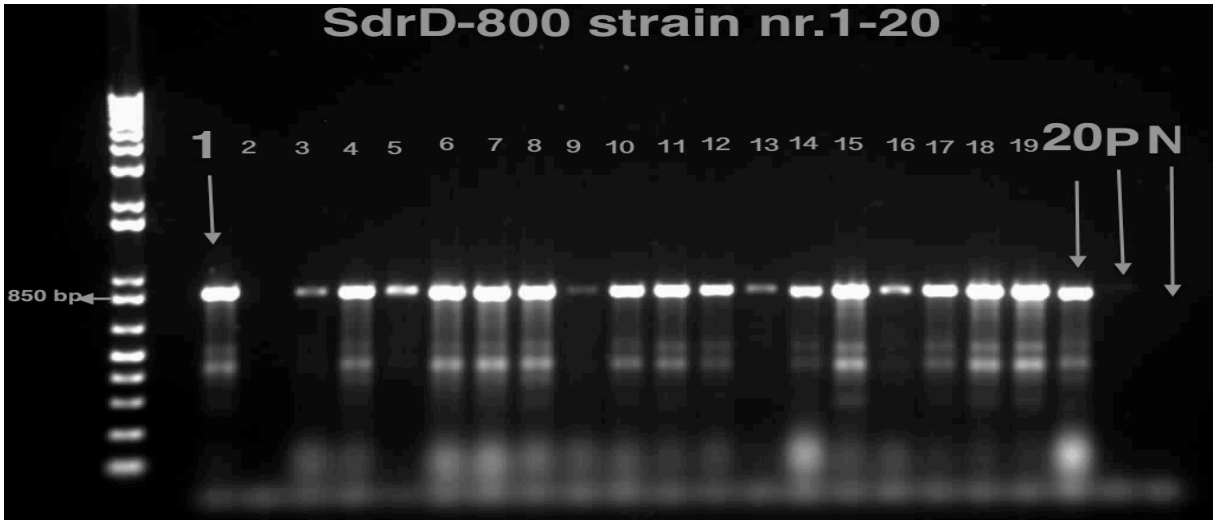

Figure 16: An image of 1% agarose gel showing the result of SdrD-800 PCR for *S. aureus* strain nr.1 to 20(from left to right), molecular weight standard (1Kb plus), in Lane "1" strain nr.1, lane "2" strain nr.2 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive control and Lane "N" negative sample that contain water instead of DNA template

SdrD-800 PCR product from DNA strain nr.21 to 51 gave one single band around at a size 850bp, whereby it was expected band at 840 bp for SdrD-800 PCR product (see Figure 17 and figure 18).

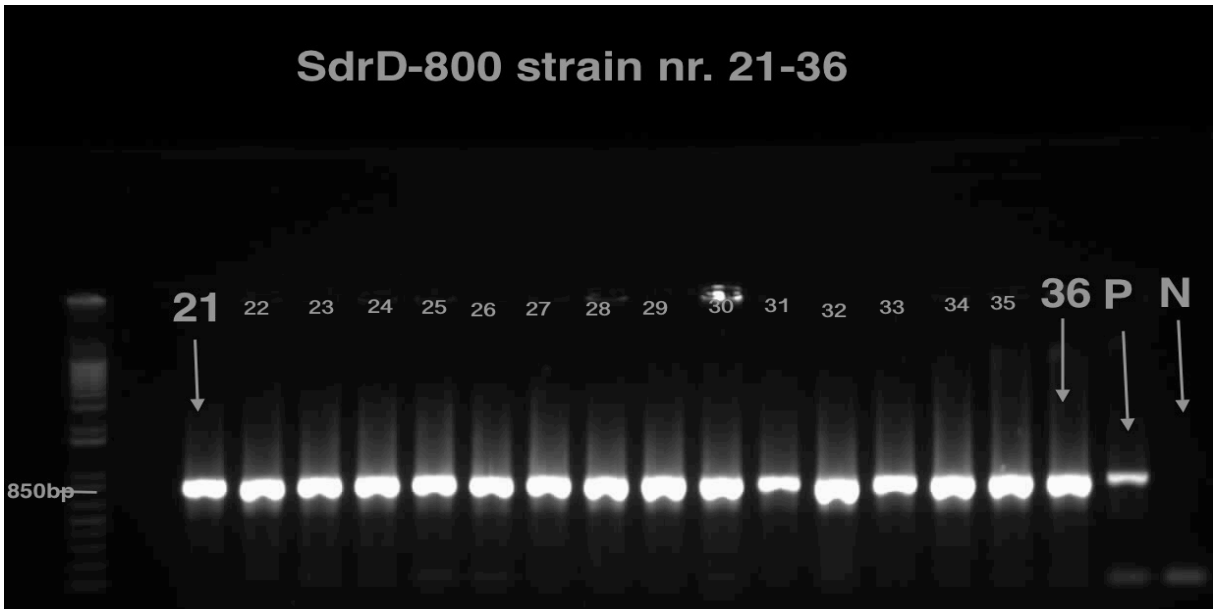

Figure 17: An image of 1% agarose gel showing the result of SdrD-800 PCR for *S. aureus* strain nr.21 to 36(from left to right), molecular weight standard (1Kb plus), in Lane "21" strain nr.21, lane "22" strain nr.22 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive control and Lane "N" negative sample that contain water instead of DNA template.

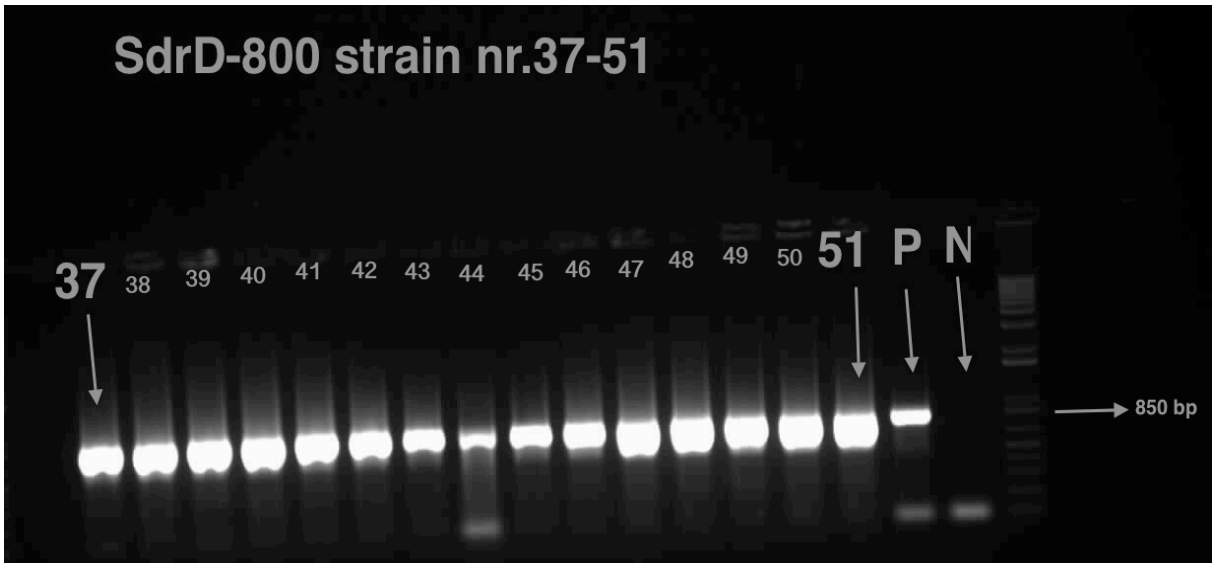

Figure 18: An image of 1% agarose gel showing the result of SdrD-800 PCR for *S. aureus* strain nr.37 to 51(from left to right), molecular weight standard (1Kb plus), in Lane "37" strain nr.37, lane "38" strain nr.38 and so on. Lane "P" *S. aureus* strain NCTC8325 for positive reference and Lane "N" negative sample that contain water instead of DNA template.

5.4 SdrD Sequence variation

sdrD A-region from all *S. aureus* strains (strain nr.1-51) were successfully sequenced, except from Strain nr.40. A total of 50 *S. aureus sdrD*-A-region sequences were aligned. Strain nr.40 was excluded from study because of the sequence was showed poor quality. The identities of sequence were checked on BLAST and sequence alignment was performed with BioEdit.

The *sdrD*-A-region variants classified into 6 groups on the basis of multiple sequence alignment (Table 14), but because of high identity similarity between SdrD1 & SdrD2 and SdrD4 & SdrD6 (Table 15), the phylogenic tree divided the variants in 4 main groups (Figure 19 & 20).

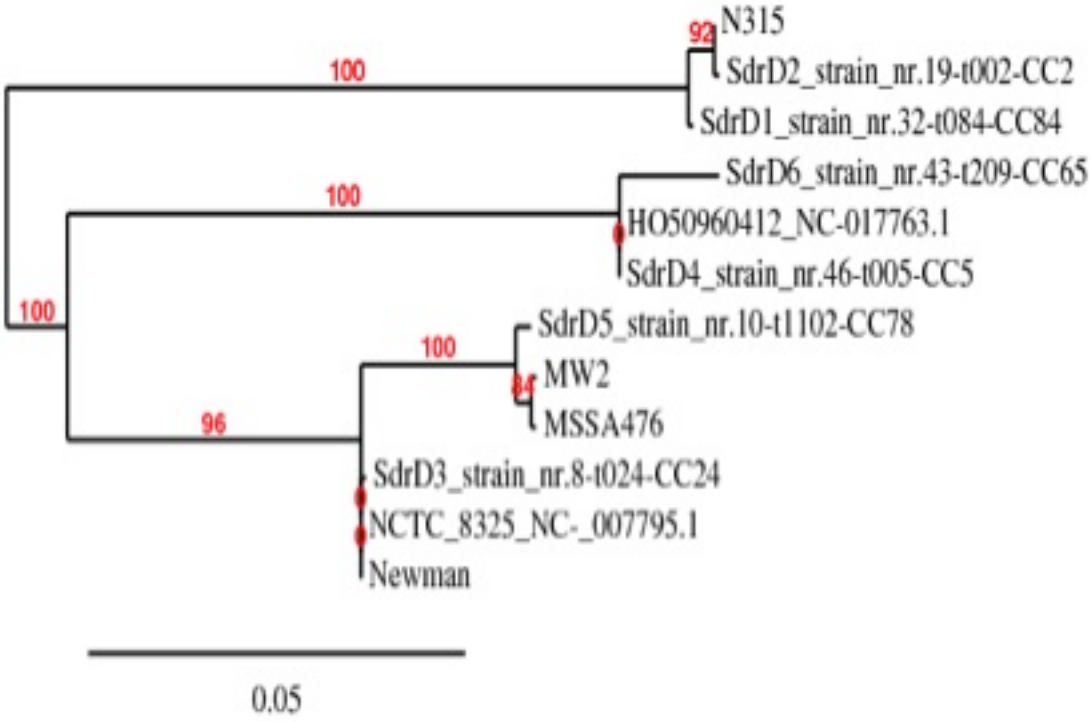

Figure 19: Maximum likelihood tree shows phylogenetic relationship among sdrD1-sdrD6 variants in selected *S. aureus* strains. Based on DNA sequence alignment. Bootstrap values shown on branches. Scale bar represents number of nucleotide changes

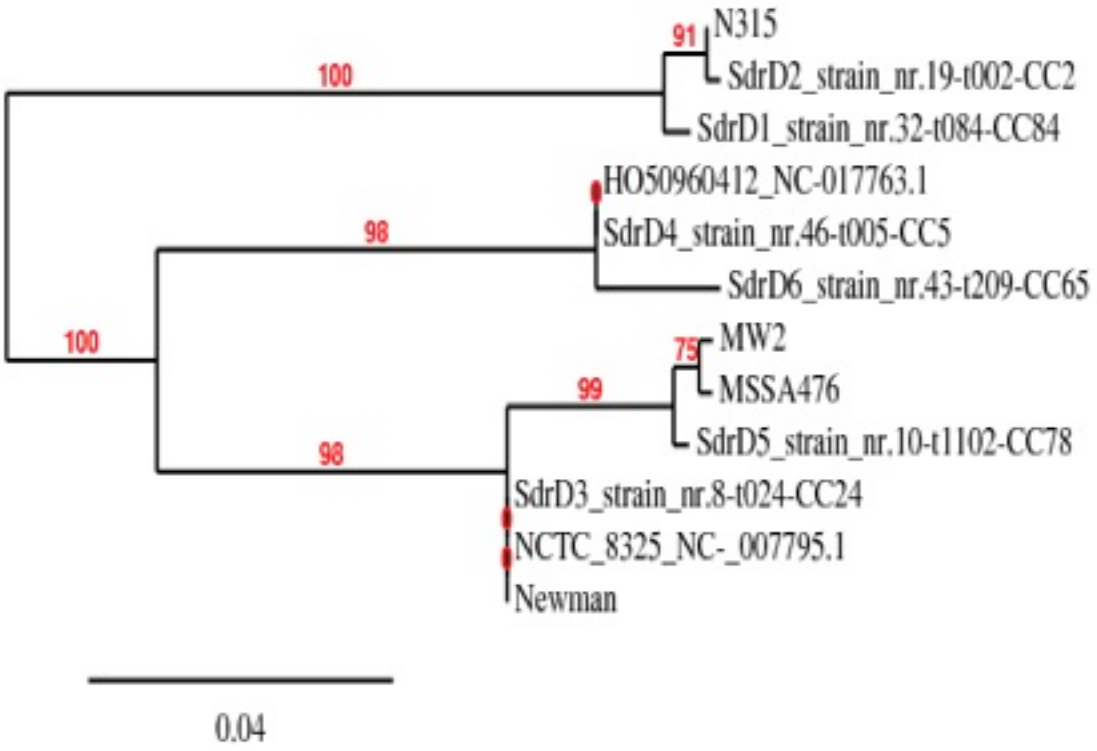

Figure 20; Maximum likelihood tree shows phylogenetic relationship among sdrD1-sdrD6 variants in selected *S. aureus* strains. Based on amino acids sequence alignment. Bootstrap values shown on branches. Scale bar represents number of nucleotide changes.

As shown in Table 14, the alignment results of *sdrD*-A-region DNA and amino acids identity indicated that there were allelic variations in *sdrD*-A-region.

Eleven strains (strains nr.1, 11, 18, 28, 29, 32, 33, 34, 37, 38, and 45) had the SdrD1variant. Fourteen strains (strains nr. 12, 14, 15, 17, 19, 23, 24, 26, 31, 41, 47, 48, 50, and 51) contained SdrD2. Seven strains (strains nr. 4, 8, 13, 20, 25, 35, and 36) had SdrD3. Five strains (strains nr. 3, 9, 16, 44, and 46) contained SdrD4. Eleven strains (strains nr. 2, 5, 6, 7, 10, 21, 22, 27, 30, 39, and 42) had SdrD5 and lastly two strains (strains nr. 43, and 49) were included in SdrD6.

The SdrD1 shared 99.4% DNA sequence identity and 98.4% amino acids sequence identity with MRSA strain N315.The SdrD2 shared 99.8% DNA sequence identity and 99.6% amino

acids sequence identity with the MRSA strain N315. The SdrD3 shared 99.9% and 99.7% DNA sequence identity and shared 99.6% and 99.2% amino acids sequence identity with those MSSA strain Newman and NCTC8325, respectively. (Table 14)
The SdrD4 shared 100% DNA sequence identity and 99,8% amino acids sequence identity with MRSA strain Ho 5096 0412. The SdrD5 shared 99.8% DNA sequence identity and 99.4% amino acids sequence identity with MRSA strain MW2 and MSSA strain MSSA476. The SdrD-A region for SdrD6 shared 98.7% DNA sequence identity and 98% amino acids sequence identity with the MRSA strain Ho 5096 0412. (Table 14)

The highest identity similarity within sequenced isolates was between SdrD1 and SdrD2 (99.6%) and between SdrD4 and SdrD6 (98.7%) .The smallest Similarities were between SdrD2 and SdrD5 (87.4%) (Table15).

Table 14 shows, the SdrD-A-region variation of DNA and amino acid sequence identity of sequenced isolates compared with different reference strains. In Table 15, the SdrD-A-region DNA sequence similarity of sequenced isolates to each other was indicated. The alignment result of SdrD-A-region DNA variation with different reference strains is shown in Appendix 4 and variation in amino acids level is shown in Appendix 5.

Table 14: The percentage identity similarity of *sdrD* variants with different *S. aureus* strains reference.

ID similarities in % with	GROUPS		SdrD1	SdrD2	SdrD3	SdrD4	SdrD5	SdrD6
	NCTC8325	DNA level	89.0	88.9	99.7	91.0	97.6	89.9
		Amino acids level	86.3	86.2	99.2	90.0	96.6	88.4
	N315	DNA level	99.4	99.8	88.9	87.3	87.1	87.4
		Amino acids level	98.4	99.6	85.9	85.5	84.5	86.3
	Ho50960412	DNA level	87.6	87.4	91.2	100	90.8	98.7
		Amino acids level	86.0	85.8	90.2	99.8	89.4	98.0
	MW2	DNA level	87.4	87.2	97.7	90.6	99.8	89.6
		Amino acids level	85.2	84.8	96.8	89.2	99.4	88.0
	MSSA476	DNA level	87.6	87.3	97.8	90.6	99.8	89.6
		Amino acids level	85.4	84.8	97.0	89.2	99.4	88.0
	Newman	DNA level	89.2	89.1	99.9	91.2	97.8	90.0
		Amino acids level	86.6	86.4	99.6	90.4	97.0	88.8

Table 15: The percentages identity similarities of *sdrD* variants in DNA level within groups

	SdrD1	SdrD2	SdrD3	SdrD4	SdrD5	SdrD6
SdrD1	100	99.6	89.2	87.6	87.6	87.6
SdrD2	99.6	100	89.0	87.5	87.4	87.6
SdrD3	89.2	89.0	100	87.6	87.6	87.6
SdrD4	87.6	87.5	87.6	100	90.8	98.7
SdrD5	87.6	87.4	87.6	90.8	100	89.8
SdrD6	87.6	87.6	87.6	98.7	89.8	100

SdrD1= strain nr.1, 11, 18, 28, 29, 32, 33, 34, 37, 38, 45

SdrD2= strain nr. 12, 14, 15, 17, 19, 23, 24, 26, 31, 41, 47, 48, 50, 51

SdrD3= strain nr. 4, 8, 13, 20, 25, 35, 36

SdrD4= strain nr. 3, 9, 16, 44, 46

SdrD5= strain nr. 2, 5, 6, 7, 10, 21, 22, 27, 30, 39, 42

SdrD6= strain nr. 43,

5.5 Cloning

To determine the role of *sdrD* variants in adhesion, adhesion assays will be performed with *L. lactis.* This required cloning of *sdrD* variants in a plasmid that can be expressed in *L. lactis*. Strain nr.32 was picked as representative for sdrD1. The full-length *sdrD* was amplified and cloned in plasmid PMG36e and then transformed into *L. lactis* by electro-transformation.

Insert preparation

Isolation of genomic DNA for stain nr.32 was done as described under "method". Full-length *sdrD* PCR was performed (Table 8). The PCR product was checked on 1 % agarose gel and gave single strong band between 3kb and 4kb (Figure 21). Whereas the band was expected at size 3.4 kb. The PCR product was purified from the gel and the concentration was measured. The concentration obtained was 18.4ng/µl and the purity ratio (260/280) was 1.84 (data not shown).

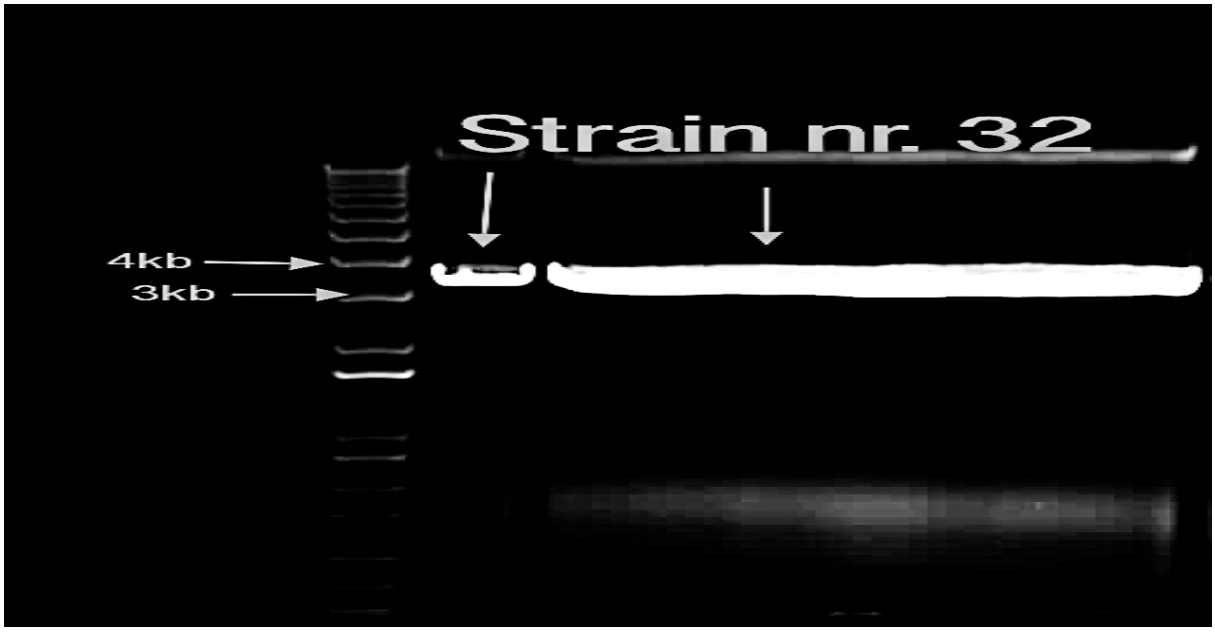

Figure 21: An image illustrated the PCR product of sdrD full length 3447 bp. Molecular weight standard (1Kb plus) used as marker.

The purified *sdrD* full length PCR product was used to perform SacI and SphI enzyme digestion (see 4.3.1). The product was checked again on 1% agarose gel after restriction enzyme digestion for specificity. The result showed a single bright band in between 3kb and 4kb (Figure 22) and the product was used for ligation (Table 9).

Figure 22: 1% agarose gel shows, molecular weight standard (1Kb plus), strain 32 after purification and restriction (SacI and SphI) enzyme digested.

Preparation of plasmid pMG36e:SdrDNCTC8325

The first strategy was to cut out sdrD_NCTC8325 from the plasmid pMG36e (Figure 4) and replaced with sdrD1 (strain nr.32). Isolation of plasmid from *L. lactis* was done as described under "method" and the concentration was measured.
The concentration was 161ng/µl and the purity (260/280) ratio was 2.03. The product (pMG36e:SdrDNCTC8325) was XbaI and XhoI enzyme digested (see procedure 4.3.2) and checked on 1% agarose gel.

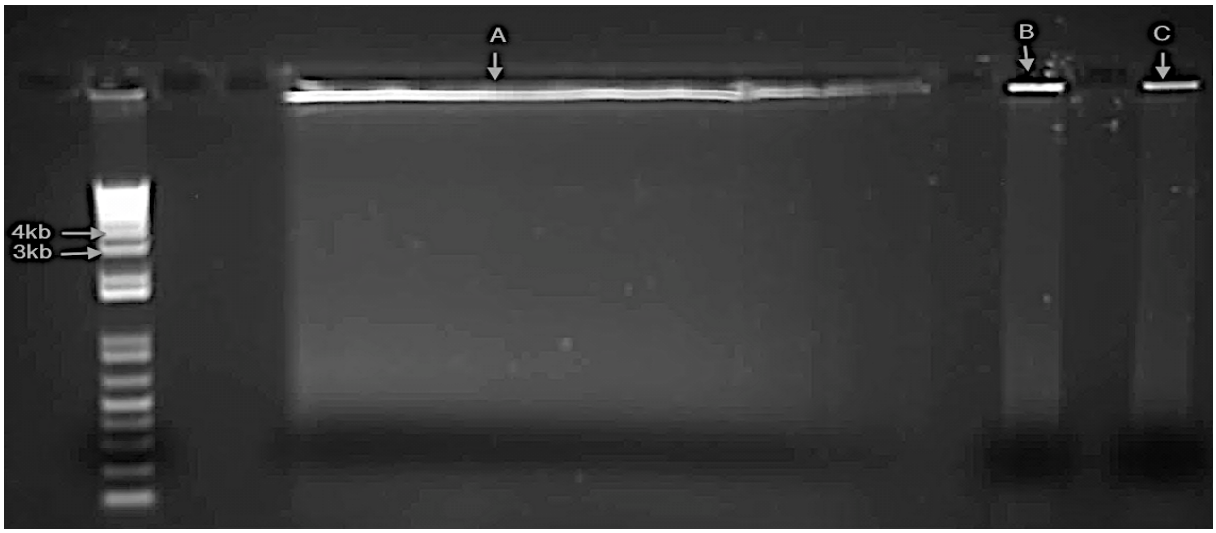

Figure 23: 1% agarose gel illustrated the result of pMG36e:SdrDNCTC8325 isolated from *L. lactis* after enzyme digest. Molecular weight standard (1Kb plus), lane A pMG36e:SdrDNCTC8325 digested by enzyme XbaI and XhoI. Lane B pMG36e:SdrDNCTC8325 digested only by enzyme XbaI and Lane C pMG36e:SdrDNCTC8325 digested only by enzyme XhoI.

As shown in Figure 23, the result of restriction enzyme digestion showed a smear band. Two single bright bands were actually expected at 3.6kb for pMG36e and at 4kb for SdrD_NCTC8325.

The plasmid DNA pMG36e:SdrDNCTC8325 (plasmid isolated from *L. lactis*) was used to transform into *E. coli*, but no colonies were obtained. The transformation and the restriction enzyme digestion failed.

Therefore the preparation of vector pMG36e:SdrDNCTC8325 was stopped and the preparation of plasmid pMG36e was proceeded.

Preparation of plasmid pMG36e

Restriction enzyme digest for pMG36e:SdrDNCTC8325 plasmid isolated from *L. lactis* and transformation of plasmid pMG36e:SdrDNCTC8325 into *E. coli* did not gave the result. Therefore, an alternative strategy was applied. That is using empty pMG36e plasmid (Figure 5). Plasmid isolation from *L. lactis* was done and the concentration was measured.

The concentration was 187ng/µl and the purity (260/280) ratio was 2.01. The product (pMG36e) that was isolated from *L. lactis* was SacI and SphI enzyme digested (see procedure on 4.3.2) and checked on 1% agarose gel. The result showed two single bands at 2 kb and 4 kb (Figure 24), the expected result was supposed to be a single bright band at 3.6 kb for pMG36e.

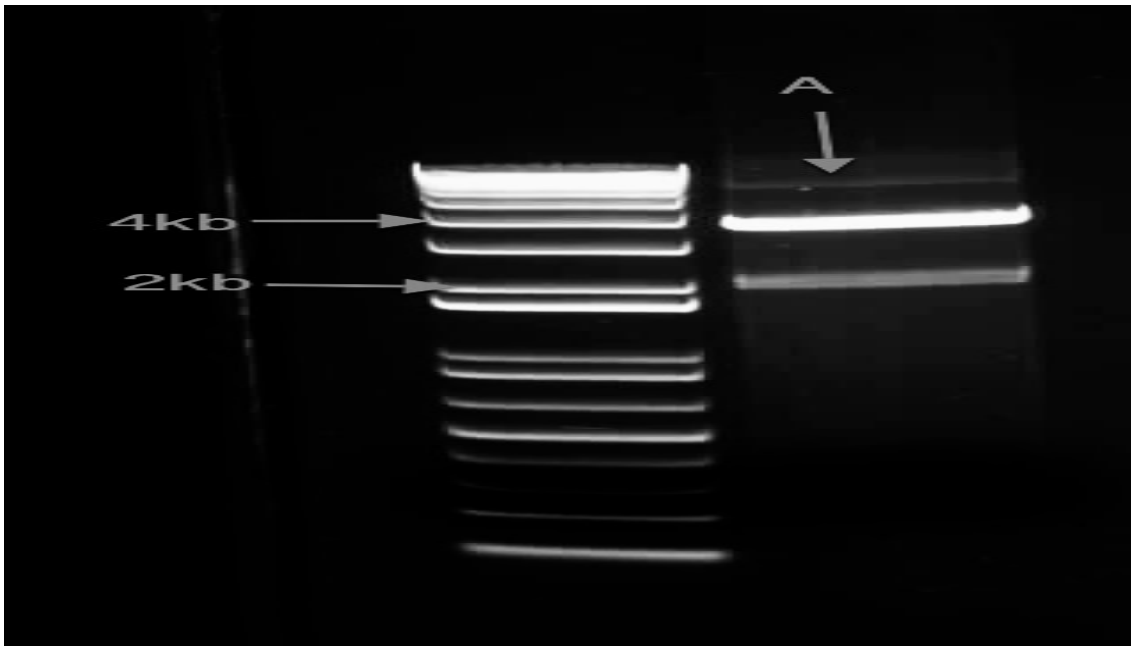

Figure 24: 1% agarose gel; molecular weight standard (1Kb plus), the result of pMG36e vector isolated from *L. lactis* (lane A) after restriction enzyme (SacI and SphI) digested.

solation of plasmid from *E. coli*

The restriction enzyme digestion of pMG36e isolated from *L. lactis* result showed two single bands at 2 kb and 4 kb (Figure 24). Even though expected result was a single bright band at 3.6kb for pMG36e.

To investigate this further the *L. lactis* DNA containing plasmid pMG36e was transformed into *E. coli.* Transformation of plasmid pMG36e into *E. coli* gave several colonies. The plasmid pMG36e was isolated from *E. coli* and the concentration was measured.

The concentration of plasmid pMG36e isolated from *E. coli* was 938.3ng/µl and purity (260/280) ratio was 2.15.

E. coli DNA containing plasmid pMG36e was SacI and SphI enzyme digested (see procedure on 4.3.2) and was checked on 1 % agarose gel. The result showed a single band at in between 3kb and 4kb as expected (Figure 25).

The product was purified from agarose gel and the concentration measured. The concentration after purification was 84.5ng/µl and purity (260/280) ratio was 2.04. This product was purified from gel for further use in ligation (Table 9).

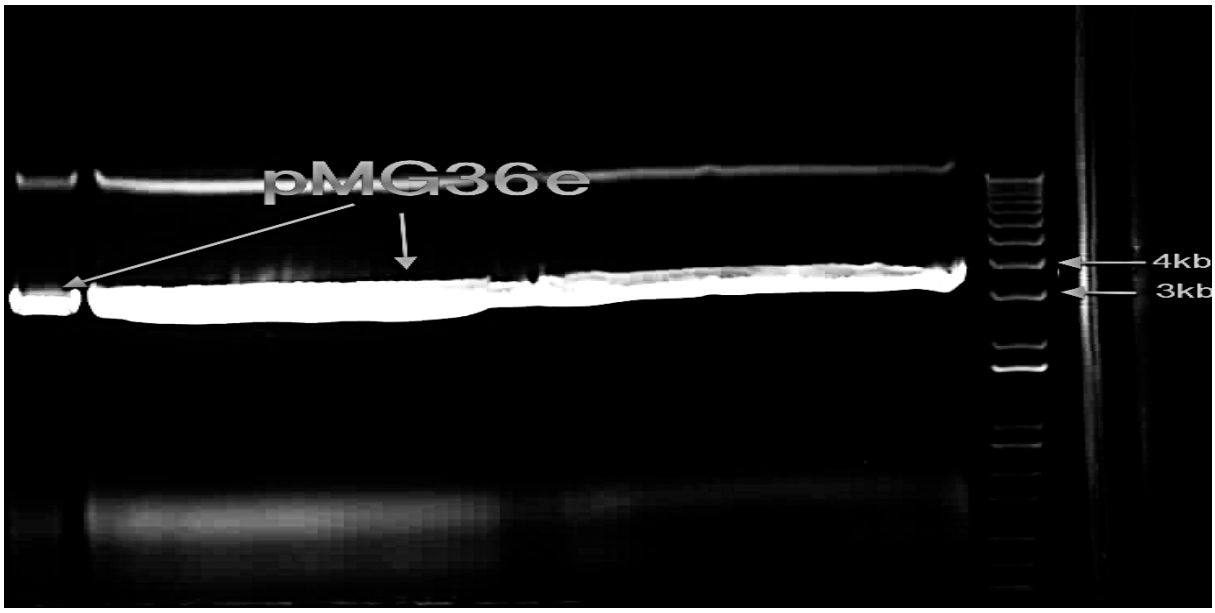

Figure 25: 1% agarose gel; molecular weight standard (1Kb plus), the result of vector pMG36e after restriction enzyme (SacI and SphI) digested.

Transformation of Ligation mix (pMG36e + SdrD1) into *E. coli*

SdrD1 insert that was purified and restriction enzyme (SacI and SphI) digested and plasmid (pMG36e) isolated from *E. coli*, restriction enzyme (SacI and SphI) digested and purified were used in a ligation mix. Ligation mix was transformed into *E. coli.*

Transformation was done with three different vector:insert ratio (see Table 9 for details). The transformation of ligation mix was successful. All of the three different vector insert ratios (1:1, 1:3 and 1:10) gave a good number of colonies. Twelve colonies from 1:3 ratio-plate screened for the right insert (see procedure on 4.3.4).
Nine colonies were SdrD positive; one colony gave double band (lane 7) and eight colonies gave single band at 4kb the result illustrated in Figure 25.
The constructs were verified by sequencing. The sequencing result showed that five (colony from lane nr. 1, 2, 3, 8 and 12 Figure 26) of eight have a pMG36e:sdrD1 constructs (Data not shown).

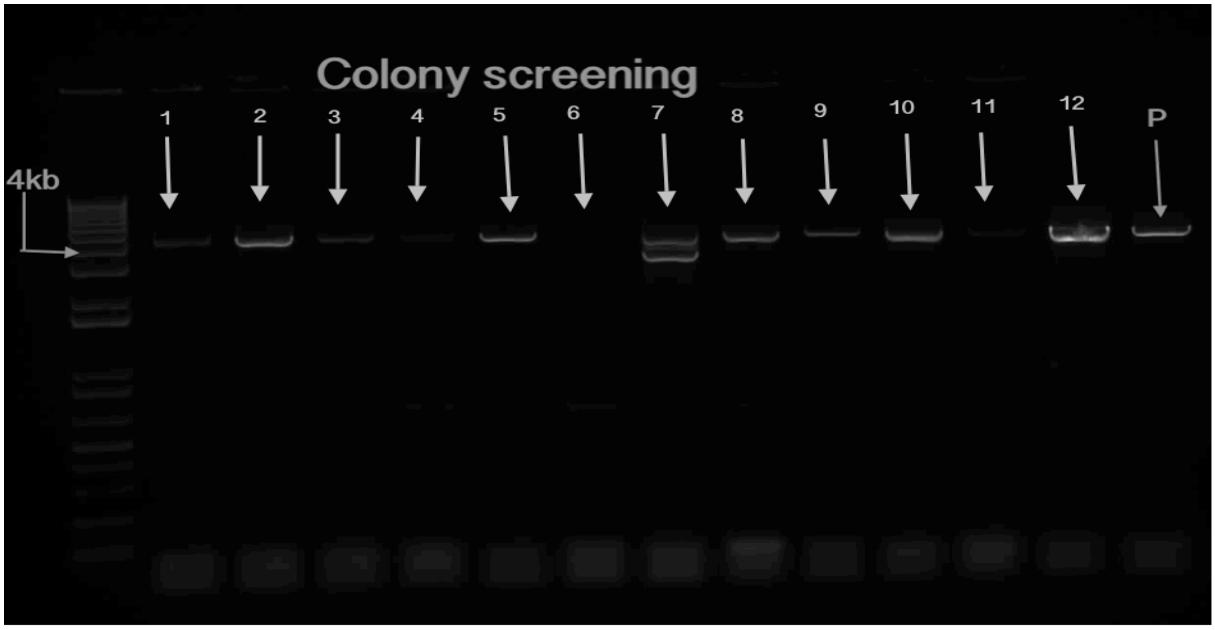

Figure 26: 1% agarose gel, molecular weight standard (1Kb plus), picture illustrating the result of colonies screening. Lane P, positive control.

5.6 Electro-transformation of pMG36e:sdrD1 into *L. lactis*

An adhesion assays will be performed with *L. lactis* + sdrD from NCTC8325, *L. lactis* +sdrD1 and *L. lactis* without SdrD, while we have the constructed plasmid in *E. coli*. Since *E. coli* is a gram-negative bacterium, it cannot use as surrogate bacteria. Therefore transferring of constructed plasmid (pMG36e:SdrD1) from *E. coli* into *L. lactis* was necessary.

In this step, attempts were made to transform pMG36e:SdrD1 from *E. coli* into *L. lactis* by electro-transformation. Two colonies were recovered on a selective media and plasmid isolation was performed. The product was screened for the right insert (see procedure on 4.3.4)**.**

Regardless of the successful transformation into *E. coli*, both obtained trans-formants were sdrD1 insert (strain nr.32) negative after electro-transformation. The expected band at a size between 3 kb and 4kb was not observed. (Data not shown)

6. Discussion

DNA from a total of 51 *S. aureus* strains was isolated, and the A-region of *sdrD* was amplified, purified and the products were sequenced. The identity of the sequence was checked using BLAST and the sequences were aligned using BioEdit program.
To investigate adhesion ability of variants, the full-length SdrD1 variant was cloned into a shuttle vector and transformed into *E. coli*, thereafter into *L. lactis*.

According to the European Centre for Disease Prevention and Control (ECDC) *S. aureus* caused 21.5 % of surgical site, 11.4 % of bloodstream infections in hospitalized patients (Zarb et al., 2012). In addition data that is collected from 23 countries showed the percentage of MRSA infection was 34.2 % (Zarb et al., 2012). This demonstrates the importance of increased understanding of the molecular aspects of interaction between *S. aureus* and host cells.
The crucial steps for the colonization and survival of bacteria depend on the ability to adhere and invade cells. Decolonization of *S. aureus* carriers might reduce postoperative staphylococcal infections. (Ten Broeke-Smits et al., 2010)

Bacterial surface proteins MSCRAMMs are important for adherence (Josefsson et al., 1998). SdrD, a MSCRAMMs family protein plays an important role in *S. aureus* adhesion and pathogenesis, but not essential for bacterial survival (Xue et al., 2011). A recent report noted that *sdrD* is associated with MRSA strains (Sabat et al., 2006, Paniagua-Contreras et al., 2014).

The study of MSCRAMMS can lead to the development of several potentially efficacious immunological therapeutic and prophylactic strategies to control *S. aureus* (Flock, 1999).

It assumed that the A domain of SdrD interact with host protein which is not yet identified (Josefsson et al., 1998). There are indications that SdrD can interact with relevant human proteins (unpublished results, HMI research group).

The surface adhesion proteins varies among *S. aureus* isolates (McCarthy and Lindsay, 2010).There may be allelic variation in SdrD that either improve or reduce its binding to host cells. For this purpose, we investigated the allelic variation of SdrD-A-region. Our results show that there is allelic variation in SdrD-A-region.

"The great number of nucleotide differences is a general characteristic of cell surface genes in *S. aureus"* (Kuhn et al., 2006). In this study Khun et al demonstrate the comparison of allelic and nucleotide divergence between conserved genes and adhesions genes of *S. aureus*. The gene diversity of adhesions genes was 10 times greater than conserved gene, and concluded the variation might be due to evolution results from gene recombination (Kuhn et al., 2006).

There are four sources of genetic variation. Spontaneous mutations, that results from errors, mistakes, accidents, and lesions. These can happen due to DNA replication or internal or external environmental influence. (Arber, 1995)

DNA recombination between slightly different genetic information brings a large variety in individual genome composition. DNA rearrangement cause genetic variation. (Arber, 1995)

Horizontal gene transformation (HGT) refers to the transfer of genes between organisms in a manner other than traditional reproduction. Many clinically important bacteria acquire virulence factor and antibiotic resistance through HGT. (Arber, 1995)

A few DNA sequence alterations bring a selective advantage to the organism, while many more sequence alterations cause a selective disadvantage or, in the extreme case, lethality (Arber, 1995). This rule indicates that, in general spontaneous genetic variations are not in response to a specific need of an organism in its environment.

A major question still remains to be answered, whether this variation is due to insertion, deletion, mutation or horizontal gene transformation.

The mechanism of how the *sdrD*-A-region variation happened is still unknown and it is not the aim of the study. Our concern is if variation contributes for ligand binding ability. If not why the bacteria used energy to change a DNA sequence, which is lead to amino acids sequence change? There are a lot of hypothesis that can arise from this result. But this study is the start and leads too many hypotheses that would be answer in future. "This is not the end, this is not even the beginning of the end, but it is perhaps the end of the beginning" Winston Churchill.

Our results show that there is allelic variation in SdrD-A-region. Similar finding was reported previously. A previous study reported several insertion and deletion mutation in the A-region

of *SdrC*, *SdrD* and *SdrE.* The DNA sequence identity of *SdrD* A-region among *S. aureus* isolated from different region shared 99.15% and B- repeats shared 99.84%. (Xue et al., 2011)

Xue et al analyzed the full-length of Sdr-families. In our study, the analyzed was done only on A-region of *sdrD*, because it presumed the A-region of *sdrD* that interact with host cells. The variation in the other region might be affecting the outcome of adhesion study. This can be restricted when the adhesion study will be done in *S. aureus*. In *S. aureus* it is possible to do knockout of the other region and figure out only the contribution of *sdrD* A- region.

Xue et al reported that, there is allelic variation in B-region of *sdrD* (Xue et al., 2011). Moreover it is described that the B-domain of Sdr-family protein SdrF in *S. epidermis* was involved in adhesion (Arrecubieta et al., 2007). If the assumption of A-region interact with host cell is not true, the variation in B-region of *sdrD* can affect the outcome of study.

It is possible that variation in some surface protein has resulted in altered ligand binding affinity. The FnBPB proteins show a high degree of variance. However the ligand binding seems to be unchanged (Burke et al., 2010).

On the other hand variation in FnBPA is associated with varying ligand-binding affinities (Loughman et al., 2008). These suggest variation in *sdrD* can result with altering ligand binding or not. If variation alters ligand binding, what are the consequences of this?

The new strategies aiming at controlling of virulence factors, to weaken bacteria without directly killing them is to develop efficacious vaccine (Papa et al., 2013). Understanding the role of allelic variation may help to identify failure of previous *S. aureus* vaccines. The inclusions of multiple allelic antigens probably result in a more effective vaccine. There is a possibility that “one size hat doesn’t fit all”. All vaccines tested and failed in clinical trials not take to account the allelic variation.

If variation alters ligand binding, it means some variants bind stronger than other; it will be hard to remove these variants. This means treatment for weak binding variants can’t work for strong binding variants. To develop efficacious vaccines, there is a need to understand the role of allelic variation in virulence factor of *S. aureus*.

Despite of the complexity of *S. aureus* and its myriad virulence factors, with deep knowledge of the critical determinants of *S. aureus* virulence factor, for example with including variant antigen in vaccine, it is not an unattainable task; there is a hope to develop successful *S. aureus* vaccine in clinical trial.

If *sdrD* A-region variations not alter ligand binding, what it means? This suggests that the variations are the result of insertion deletion and shows the instability of the SdrD protein. This leads to engaging SdrD as vaccine antigen doubtful. Because instable proteins are not a good antigen (Foster and Hook, 1999).

If variations not alter ligand binding, it may affect bacterial condition of infection. MRSA share a common genetic background with MSSA and the success of MRSA is due to extrinsic and genetic determinants of the bacterium (Hallin et al., 2008).

We found *sdrD* A-region sequence from MSSA strains that match with MRSA strains *sdrD* A-region reported in GenBank. The MSSA *sdrD* identity matching with MRSA strains probably the precondition of *S. aureus* evolution or it is due to environmental and host factor stress. This might be affecting the outcome of infection.

Interestingly an association between *sdrD*-A region variation and Spa type was observed in our study. Spa typing is a typing method for *S. aureus*, based on sequencing of a single polymorphic variable-number tandem repeat (VNTR) region of the *S. aureus* protein-A gene (Sangvik et al., 2011).

In Tromsø 6 study the second most common spa type was t084, CC084 (7.6%) (Sangvik et al., 2011). In our study the SdrD1 variant seemed to be associated with CC084, SdrD3 with CC24, SdrD4 with CC5, and SdrD5 with CC78. Except for SdrD2 variant that belonged to different clonal complex (CC2 and CC84). Based on this, one could speculate that may be every clonal complex have own SdrD type.

One factor that is not taken into account in this study is the possibility of sequencing error. The majority of strains are sequenced once but a few strains are sequenced twice. Since both strands were sequenced bidirectional, the probability of sequencing error was very little and the results are repeatable.

PCR

In order to found out the allelic variation in *sdrD* A-region, it was necessary to do PCR and sequencing. For this aim, it was important to optimize the PCR. Two control strains, *S. aureus* strain MSSA476 and NCTC8325 were used to optimize PCR.

Under optimization of annealing temperature in the gradient PCR strain NCTC8325 gave single band at 60.4 °C, strain MSSA476 gave multiple bands at this temperature (see Figure 11 and 12). This could be due to the difference of the concentration of DNA or could be the strains characteristic is different, or both reasons were contribute to the observed difference. The concentration was almost four times higher for DNA from strain MSSA476 (99.46ng/µl) than DNA from strain NCTC8325 (24.46ng/µl).

There was also an unexpected event during optimization of annealing temperature. According to the theory of PCR, when the temperature (50°C) is reduced under the melting point of primers, the primer may bind non-specifically and non-specific bands seen on a gel. In this case this principle wasn't occurring. The band was weak single band in low temperature (see figure 11).

After optimization was done, PCR for DNA from the first 20 *S. aureus* isolates from Tromsø 6 study was done. For both PCR (sdrD-700 & sdrD-800), most strains were revealed multiple bands in addition to single band at a right size (see figure 13 & 16). In spite of multiple bands on 1% agarose gel, strains were gave legible sequence result. The reason of this problem was unknown until we were started to use new batch "reddymix" for DNA from the next 31 *S. aureus* isolates from Tromsø 6.

The same DNA template gave a good gel picture after changing "Reddymix" (new batch). The product was from the same manufacturer; the only difference was manufacturing day and different batch number.

Cloning

Because *S. aureus* expresses different combination of surface proteins, it is difficult to figure out the contribution of single protein. This approach was complemented by the expression of SdrD protein separately on the surface of the surrogate gram-positive bacterium *L. lactis*. The expression of sdrD A-region on the surface of *L. lactis* was impossible due to the lack of signal sequence, cell wall-spaning segment and membrane spanning region. As a result full-length sdrD was cloned in shuttle plasmid.

The adhesion method was based on previous study (Barbu et al., 2010, Kintarak et al., 2004). The principle is to let *L. lactis* with PMG36e:SdrD1, *L. lactis* with PMG36e, and *L. lactis* without plasmid adhere with HEK293 cells, which is transfected with labeled eukaryotic partner proteins, and un-transfected cells. Then determine the difference in adherence.

To do adhesion assay, cloning should be performed. Full SdrD was amplified and cloned in plasmid pMG36e and transformed into *L. lactis* by electro-transformation.

The first strategy was to cut out sdrD-NCTC8325 from the plasmid pMG36e:sdrD_NCTC8325 (see figure 4) and substituted that with one of sdrD variant.

Neither restriction enzyme digestion nor transformation into *E. coli* was successful. Restriction enzyme cut result was not effective (see figure 23) and transformation didn't yield any transformants.

Unsuccessful transformation into *E. coli* was unexpected, as this method (standard transformation method) has been found to efficiently introduce plasmid DNA into *E. coli*. In principle, shuttle plasmid can be moved back and forth between different hosts (Griffiths et al., 1999).

E. coli competent cells used in this experiment were found to be good quality and that the positive control which included in the experiment was illustrated that the method also worked efficiently.
The restriction enzymes were tested for effectiveness by using another plasmid DNA (not mentioned under method) and the enzymes were adequate. Isolation method works on the same plasmid DNA without *sdrD* and the method was good enough. This led to the assumption that the lack of success with the experiment could be pMG36e:SdrDNCTC8325. But what it could be? Maybe the plasmid prepared to use as it is, or some modification done by *L. lactis*?

Because of these problems the strategy was changed. The second strategy was to use pMG36e without sdrD (see figure 5). Restriction enzyme digestion on this plasmid also failed (see figure 24), but transformation into *E. coli* succeeded.
The possible explanation for unsuccessful enzyme digestion on plasmid can be genetic modification was done by *L. lactis* that make a recognition sequence unrecognized for restriction enzymes, most probably methylation?

Electro-transformation
Before we could start the adhesion assays transferring of constructed plasmid from *E. coli* into *L. lactis* was required. This was done with electro transformation. Electro-transformation of construct plasmid into *L. lactis* was failed.

Despite of the successful construct and transformation into *E. coli* with standard method, the colony obtained after electro-transformation was negative and the adhesion study could not be continued.
Electro transformation and preparation of electro competent cells was done twice without success. In the second attempt, the transformation was done with different plasmid DNA concentration and didn't work.

The limitation under electro-transformation was the positive and negative controls weren't included in the experiment. Without appropriate positive and negative controls for transformation reactions, it was very difficult to evaluate the problem was in preparation of electro competent cells, efficiency of competent cells or transformation process.

Troubleshooting was virtually impossible without any controls. Because of time limit the experiment was not repeated; no investigation was done to figure out the possible explanation.

It could be in electro-competent cell preparing steps, *L. lactis* strain M1363 was not grow fast to the OD which is necessary to get best result, and freezing step with liquid hydrogen or dry ice/ethanol was not performed.

L. lactis strain M1363 took 10 hours to grow to appropriate OD, whereas expected time was 4 hours. It is recommended that the fast growing cells usually give the best result and the freezing step is increasing competence efficiency of the cells.

7. Conclusion and future study

There is allelic variation in A-region of SdrD. For further study of adhesions, the constructs (pMG36e:SdrD1 in *E. coli*) was prepared, and constructing of the other variants is ongoing in the laboratory of HMI research group.

For further research a test should be done to assure if variation can alter ligand binding affinity or not, if it alters binding affinity what is the consequence of that? It is important to consider how much divergence is required for variants to function differently. It is also important to find out if there is variation in other regions of *sdrD*, and if they can affect the adhesion study. These questions need further investigation before "engaging" SdrD for future therapy target. It is very important to address future studies to determine the pathogenic potential of allelic variants.

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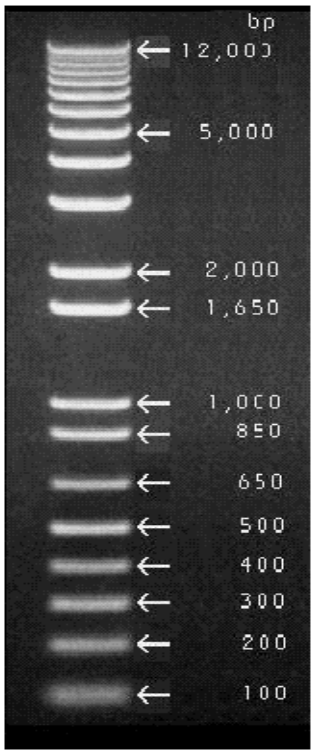
Appendices

Appendix 1: Overview of bacteria isolate

ID[a]	T6[b] ID	spa type	CC[c]	ID	T6 ID	spa type	CC
1	61010014	t084	84	27	6101017	t5257	78
2	61010019	t5449	78	28	6101018	t084	84
3	61010021	t223	5	29	6101020	t084	84
4	61010026	t305	24	30	6101020	t814	78
5	61010037	t759	78	31	6101020	t2119	84
6	61010045	t167	78	32	6101021	t084	84
7	61010051	t349	78	33	6101021	t084	84
8	61010060	t024	24	34	6101021	t084	84
9	61010103	t3610	S	35	6101021	t024	24
10	61010105	t1102	78	36	6101022	t024	24
11	61010109	t084	84	37	6101022	t084	84
12	61010111	t601	2	38	6101022	t094	84
13	61010112	t1476	24	39	6101023	t280	78
14	61010116	t045	2	40	6101024	t2103	153
15	61010122	t002	2	41	6101024	t084	84
16	61010128	t005	5	42	6101026	t078	78
17	61010132	t581	2	43	6101026	t209	65
18	61010135	t5232	84	44	6101026	t309	5
19	61010136	t002	2	45	6101028		
20	61010146	t008	24	46	6101030	t005	5
21	61010150	t528	E	47	6101030	t084	84
22	61010164	t1102	78	48	6101030	t084	84
23	61010165	t084	84	49	6101030	t186	S
24	61010168	t4173	267	50	6101030	t084	84
25	61010169	t008	24	51	6101030	t548	2
26	61010173	t5423	2				

a= Strain ID in thesis
b= Tromsø 6 study ID
c= clonal complex

Appendix 2: Marker that is used in this study



1 Kb PLUS DNA LADDER

Appendix 3: Buffer and reagent Composition

1x ReddyMix

0,625 units	ThermoPrime Taq DNA polymerase
75mM	Tris-HCL (pH 8,8)
20mM	$(NH_4)_2SO_4$
1,5mM	$MgCl_2$
0,01%(v/v)	Tween 20
0,2mM	each of dATP, dCTP, dGTP, dTTP

Precipitant and red dye for electrophoresis

TE-Buffer pH: 1x

10mM	Tris-Hcl pH: 8
0,5M	EDTA

TAE-buffer 1x

40mM	Tris-Hcl (Trizma –base)
20mM	acetic acid
1mM	EDTA pH:8

1x NEBuffer 4:

50 mM	Potassium acetate
20mM	Tris acetate
10 mM	Magnesium acetate
1mM	DTT pH 7.9

SGM17-Gly medium

0.5MSucrose + 0.5% Glucose + 2.5%glycine

SGM17-medium with 10µg/ml erythromycin

0.5% Sucrose + 0.4% glucose +10µg/ml

Electrotransformation solution (ES)

30% Polethyleneglycol + 10%glycerol +
double distilled water

Washing Solution

0.5M Sucrose +10%glycerol + double distilled
water

BSA (Bovine Serum Albumin) 10x

2mM KPO_4 (pH 7.0 at 25°C),
5 mM NaCl
0.01 mM EDTA
0.5% Glycerol.

Lysogenic Broth (Luria broth)

1.0 % Tryptone
0.5 % Yeast extract
1.0 % Sodium chloride
400µg/ml Erythromycin

SOC

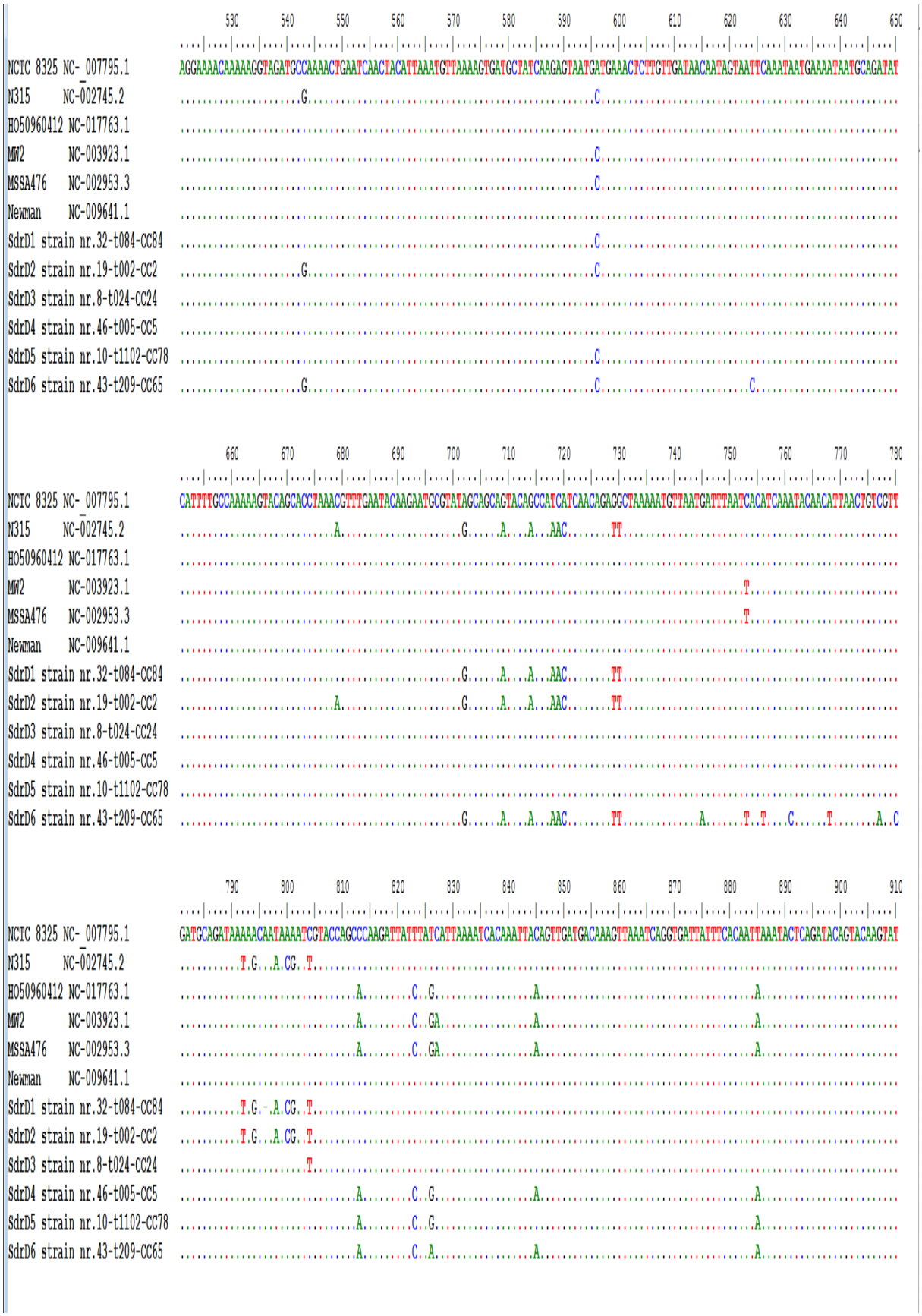
0.5% Yeast
2% Tryptone
10mM NaCl
2.5mM KCl
20mM Glucose
10mM $MgCl_2$
10mM MSo_4

Appendix 4: Variation in DNA level

```
                                 140       150       160       170       180       190       200       210       220       230       240       250       260
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1           ------------------------CAGAAAGTACTAATAAAGAATTGAACGAAGCGACAACTTCAGCAAGTGATAATCAATCGAGTGATAAAGTTGATATGCAGCAACTAAATCAAGAAGACAATAC
N315      NC-002745.2            ------------------------........................................................................................
H050960412 NC-017763.1           ------------------------........................................................................................
MW2       NC-003923.1            ------------------------........................................................................................
MSSA476   NC-002953.3            ------------------------........................................................................................
Newman    NC-009641.1            ------------------------........................................................................................
SdrD1 strain nr.32-t084-CC84     ------------------------.................-......................................G.......................
SdrD2 strain nr.19-t002-CC2      ------------------------........................................................................................
SdrD3 strain nr.8-t024-CC24      ------------------------........................................................................................
SdrD4 strain nr.46-t005-CC5      ------------------------.................-......................................................................
SdrD5 strain nr.10-t1102-CC78    ------------------------.......................................................................A................
SdrD6 strain nr.43-t209-CC65     ------------------------........................................................................................

                                 270       280       290       300       310       320       330       340       350       360       370       380       390
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1           TAAAAATGATAATCAAAAAGAAATGGTATCATCTCAAGGTAATGAAACGACTTCAAATGGGAATAAATTAATAGAAAAAGAAAGTGTACAATCTACCACTGGAAATAAAGTTGAAGTTTCAACTGCCAAA
N315      NC-002745.2            ........................................................C...............................................
H050960412 NC-017763.1           ........................................................................................................
MW2       NC-003923.1            ........................................................C...............................................
MSSA476   NC-002953.3            ........................................................C...............................................
Newman    NC-009641.1            ........................................................................................................
SdrD1 strain nr.32-t084-CC84     ........................................................C...............................................
SdrD2 strain nr.19-t002-CC2      ........................................................C...............................................
SdrD3 strain nr.8-t024-CC24      ........................................................................................................
SdrD4 strain nr.46-t005-CC5      ........................................................................................................
SdrD5 strain nr.10-t1102-CC78    ........................................................C...............................................
SdrD6 strain nr.43-t209-CC65     ........................................................................................................

                                 400       410       420       430       440       450       460       470       480       490       500       510       520
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1           TCAGATGAGCAAGCTTCACCAAAATCTACGAATGAAGATTTAAACACTAAACAAACTATAAGTAATCAAGAAGCGTTACAACCTGATTTGCAAGAGAATAAATCAGTGGTAAATGTTCAACCAACTAATG
N315      NC-002745.2            ...................................................G...............T...........................................
H050960412 NC-017763.1           ........................................................................................................
MW2       NC-003923.1            ...............................................................................C........................
MSSA476   NC-002953.3            ...............................................................................C........................
Newman    NC-009641.1            ........................................................................................................
SdrD1 strain nr.32-t084-CC84     ...............................................................................C........................
SdrD2 strain nr.19-t002-CC2      ...................................................G...............T...........................................
SdrD3 strain nr.8-t024-CC24      ........................................................................................................
SdrD4 strain nr.46-t005-CC5      ........................................................................................................
SdrD5 strain nr.10-t1102-CC78    ...................................A...............................C........................
SdrD6 strain nr.43-t209-CC65     ........................................................................................................
```


NCTC 8325 NC- 007795.1
N315 NC-002745.2
HO50960412 NC-017763.1
MW2 NC-003923.1
MSSA476 NC-002953.3
Newman NC-009641.1
SdrD1 strain nr.32-t084-CC84
SdrD2 strain nr.19-t002-CC2
SdrD3 strain nr.8-t024-CC24
SdrD4 strain nr.46-t005-CC5
SdrD5 strain nr.10-t1102-CC78
SdrD6 strain nr.43-t209-CC65

```
                                   920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1             ATGGATTGAATCCGGAAGATATTAAAAATATTGGTGATATTAAAGATCCAAATAATGGTGAAACAATTGCGACTGCAAAACATGATACTGCAAATAATTTAATTACATATACATTTACAGATTATGTTGA
N315      NC-002745.2              ........................................................................................................................................
H050960412 NC-017763.1             ...............................A........................................................................................A..
MW2       NC-003923.1              ...............................A........................................................................................C..
MSSA476   NC-002953.3              ...............................A........................................................................................C..
Newman    NC-009641.1              ........................................................................................................................................
SdrD1 strain nr.32-t084-CC84       ........................................................................................................................................
SdrD2 strain nr.19-t002-CC2        ........................................................................................................................................
SdrD3 strain nr.8-t024-CC24        ........................................................................................................................................
SdrD4 strain nr.46-t005-CC5        ...............................A........................................................................................A..
SdrD5 strain nr.10-t1102-CC78      ...............................A........................................................................................C..
SdrD6 strain nr.43-t209-CC65       ...............................A........................................................................................A..

                                   1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1             TCGATTTAATTCTGTACAAATGGGAATTAATTATTCAATTTATATGGATGCTGATACAATTCCTGTTAGTAAAAACGATGTTGAGTTTAATCTTACGATAGGTAATACTACAACAAAAACAACTGCTAAC
N315      NC-002745.2              ............A..A.......T........C................A..............GAC..G..A......CCT....G..A..T..T..A...CAA.TT....CT.....A..AG.T
H050960412 NC-017763.1             .A......................................................................................................................
MW2       NC-003923.1              .A......................................................................................................................
MSSA476   NC-002953.3              .A......................................................................................................................
Newman    NC-009641.1              ........................................................................................................................................
SdrD1 strain nr.32-t084-CC84       ............A..A.......T........C................A..............GAC..G..A......CCT....G..A..T..T..A...CAA.TT....CT.....A..AG.T
SdrD2 strain nr.19-t002-CC2        ............A..A.......T........C................A..............GAC..G..A......CCT....G..A..T..T..A...CAA.TT....CT.....A..AG.T
SdrD3 strain nr.8-t024-CC24        ........................................................................................................................................
SdrD4 strain nr.46-t005-CC5        .A......................................................................................................................
SdrD5 strain nr.10-t1102-CC78      .A......................................................................................................................
SdrD6 strain nr.43-t209-CC65       .A......................................................................................................................

                                   1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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H050960412 NC-017763.1             ......................TCT.GA..T..T.....T..A..T.....A..T........T..........CA..T...GC....G.C..G..T.......T...G..TG...................
MW2       NC-003923.1              ........................................................................................................A...........
MSSA476   NC-002953.3              ........................................................................................................................................
Newman    NC-009641.1              ........................................................................................................................................
SdrD1 strain nr.32-t084-CC84       ..CACT.....G.C....AAA.A.GC...C..T........A........T..T..A..G........T.....A......GTT...G.C..T..T........C..GGTAG.A.....T.....TAT..
SdrD2 strain nr.19-t002-CC2        ..CACT.....G.C....AAA.A.GC...C..T........A........T..T..A..G........T.....A......GTT...G.C..T..T........C..GGTAG.A.....T.....TAT..
SdrD3 strain nr.8-t024-CC24        ........................................................................................................................................
SdrD4 strain nr.46-t005-CC5        ......................TCT.GA..T..T.....T..A..T.....A..T........T..........CA..T...GC....G.C..G..T.......T...G..TG...................
SdrD5 strain nr.10-t1102-CC78      ........................................................................................................................................
SdrD6 strain nr.43-t209-CC65       ......................TCT.GA..T..T.....T..A..T.....A..T........T..........CA..T...GC....G.C..G..T.......T...G..TG...................
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                                   1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1             AAAATTCTTTAACAAATGCCAAACTAAAAGTTCAAGCTTACCACTCAAGTTATCCTAATAATATCGGGCAAATAAATAAAGATGTAACAGATATAAAAATATATCAAGTTCCTAAAGGTTATACATTAAA
N315       NC-002745.2             .T..GGA....A.GG...T..GT........G....G.....TC.G.AA.....A.C......T..T.....T...C..A...T...A..............GT......G....A........G..
H050960412 NC-017763.1             ....AA............T...T........G.......T..TAAGGA.......G.C...G.A..T............A.......TA.A..T.....T.......CA..A....AC...GTT.....
MW2        NC-003923.1             ..................................................................G.....................................................
MSSA476    NC-002953.3             ..................................................................G.....................................................
Newman     NC-009641.1             ....................................................................................................................
SdrD1 strain nr.32-t084-CC84       .T..GGA....A.GG...T..GT........G....G.....TC.G.AA.....A.C......T..T.....T...C..A...T...A..............GT......G....A........G..
SdrD2 strain nr.19-t002-CC2        .T..GGA....A.GG...T..GT........G....G.....TC.G.AA.....A.C......T..T.....T...C..A...T...A..............GT......G....A........G..
SdrD3 strain nr.8-t024-CC24        ....................................................................................................................
SdrD4 strain nr.46-t005-CC5        ....AA............T...T........G.......T..TAAGGA.......G.C...G.A..T............A.......TA.A..T.....T.......CA..A....AC...GTT.....
SdrD5 strain nr.10-t1102-CC78      ..................................................................G.....T.............................................
SdrD6 strain nr.43-t209-CC65       ....AA............T...T........G.......T..TAAGGA.......G.C...G.A..T............A.......TA.A..T.....T.......CA..A....AC...GTT.....

                                   1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1             TAAAGGATACGATGTGAATACTAAAGAGCTTACAGATGTAACAAATCAATACTTGCAGAAAATTACATATGGCGACAACAATAGCGCTGTTATTGATTTTGGAAATGCAGATTCTGCTTATGTTGTAATG
N315       NC-002745.2             .........T..C..T........T..TT.GGT...C.....TG..G...TTAAAA.T.....G..G.....ATCA..TC.A..T.T.AA.C..........TG..ATTACA..A..A...........
H050960412 NC-017763.1             C.....T..T.....T.....A..TC.A....T......G..TG.A....TTAAAG.T..G........C..AACT..TG.C..T.TAAA.G..........T.GCATTA.CAA.T.......A..T...
MW2        NC-003923.1             ....................................................................................................................
MSSA476    NC-002953.3             ....................................................................................................................
Newman     NC-009641.1             ....................................................................................................................
SdrD1 strain nr.32-t084-CC84       .........T..C..T........T..TT.GGT...C.....TG..G...TTAAAA.T.....G..G.....ATCA..TC.A..T.T.AA.C..........TG..ATTACA..A..A...........
SdrD2 strain nr.19-t002-CC2        .........T..CA.T........T..TT.GGT...C.....TG..G...TTAAAA.T.....G..G.....ATCA..TC.A..T.T.AA.C..........TG..ATTACA..A..A...........
SdrD3 strain nr.8-t024-CC24        ....................................................................................................................
SdrD4 strain nr.46-t005-CC5        C.....T..T.....T.....A..TC.A....T......G..TG.A....TTAAAG.T..G........C..AACT..TG.C..T.TAAA.G..........T.GCATTA.CAA.T.......A..T...
SdrD5 strain nr.10-t1102-CC78      ....................................................................................................................
SdrD6 strain nr.43-t209-CC65       C.....T..T.....T.....A..TC.A....T......G..TG.A....TTAAAG.T..G........C..AACT..TG.C..T.TAAA.G..........T.GCATTA.CAA.T.......A..T...

                                   1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680      1690
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325 NC- 007795.1             GTTAATACAAAATTCCAATATACAAATAGCGAAAGCCCAACACTTGTTCAAATGGCTACTTTATCTTCAACAGCTAATAAATCCGTTTCTACTGGCAAT----------------------------
N315       NC-002745.2             ....................................................................................................----------------------------
H050960412 NC-017763.1             ..GG..........TG...T.....C...T.....T...................A.....GA....TGAT..A..C.G...................----------------------------
MW2        NC-003923.1             ........................C...........................T...A...AG....GACAA..G......T.CC..A.TG..T...----------------------------
MSSA476    NC-002953.3             ........................C...........................T...A...AG.....ACAA..G......T.CC..A.TG..T...----------------------------
Newman     NC-009641.1             ....................................................................................................----------------------------
SdrD1 strain nr.32-t084-CC84       ....................................................................................................----------------------------
SdrD2 strain nr.19-t002-CC2        ....................................................................................................----------------------------
SdrD3 strain nr.8-t024-CC24        ....................................................................................................----------------------------
SdrD4 strain nr.46-t005-CC5        ..GG..........TG...T.....C...T.....T...................A.....GA....TGAT..A..C.G...................----------------------------
SdrD5 strain nr.10-t1102-CC78      ........................C...........................T...A...AG....GACAA..G......T.CC..A.TG..T...----------------------------
SdrD6 strain nr.43-t209-CC65       ..GG..........TG...T.....C...T.....T...................A.....GA....TGAT..A..C.G...................----------------------------
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Appendix 5: variation in Amino acids level

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                                   10        20        30        40        50        60        70        80        90       100       110       120       130
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325                 MLNRENKTAITRKGMVSNRLNKFSIRKYTVGTASILVGTTLIFGLGNQEAKAAESTNKELNEATTSASDNQSSDKVDMQQLNQEDNTKNDNQKEMVSSQGNETTSNGNKLIEKESVQSTTGNKVEVSTAK
N315        NC-002745.2   ------------------------------------------------X.......................................................S.......................
H050960412 NC-017763.1    ------------------------------------------------X.......................................................................................
MW2         NC-003923.1   ------------------------------------------------X.......................................................S.......................
MSSA476     NC-002953.3   ------------------------------------------------X.......................................................S.......................
Newman      NC-009641.1   ------------------------------------------------X.......................................................................................
SdrD1 strain nr.32-t084-CC84 ------------------------------------------------X....X.............G.................................S.......................
SdrD2 strain nr.19-t002-CC2  ------------------------------------------------X.......................................................S.......................
SdrD3 strain nr.8-t024-CC24  ------------------------------------------------X.......................................................................................
SdrD4 strain nr.46-t005-CC5  ------------------------------------------------X....X..................................................................................
SdrD5 strain nr.10-t1102-CC78 ----------------------------------------------X.......................K...............................S.......................
SdrD6 strain nr.43-t209-CC65 ------------------------------------------------X.......................................................................................

                                  140       150       160       170       180       190       200       210       220       230       240       250       260
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325                 SDEQASPKSTNEDLNTKQTISNQEALQPDLQENKSVVNVQPTNEENKKVDAKTESTTLNVKSDAIKSNDETLVDNNSNSNNENNADIILPKSTAPKRLNTRMRIAAVQPSSTEAKNVNDLITSNTTLTVV
N315        NC-002745.2   ......................G....L.................................A.......................S.....M..I..N..DS...............
H050960412 NC-017763.1    ....................................................................................................................................
MW2         NC-003923.1   ...............................A.......................A....................................................................
MSSA476     NC-002953.3   ...............................A.......................A....................................................................
Newman      NC-009641.1   ....................................................................................................................................
SdrD1 strain nr.32-t084-CC84 ..............................A.......................A.............................M..I..N..DS...............
SdrD2 strain nr.19-t002-CC2  ......................G....L.................................A.......................S.....M..I..N..DS...............
SdrD3 strain nr.8-t024-CC24  ....................................................................................................................................
SdrD4 strain nr.46-t005-CC5  ....................................................................................................................................
SdrD5 strain nr.10-t1102-CC78 ..............................A.......................A....................................................................
SdrD6 strain nr.43-t209-CC65 ....................................................................A.............................M..I..N..DS...N....T......

                                  270       280       290       300       310       320       330       340       350       360       370       380       390
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NCTC 8325                 DADKNNKIVPAQDYLSLKSQITVDDKVKSGDYFTIKYSDTVQVYGLNPEDIKNIGDIKDPNNGETIATAKHDTANNLITYTFTDYVDRFNSVQMGINYSIYMDADTIPVSKNDVEFNVTIGNTTTKTTAN
N315        NC-002745.2   ...NSKT.....................................................................K...............D.K..P.S.....QI.T...D
H050960412 NC-017763.1    ...............A.....K.....................................................................................................
MW2         NC-003923.1   ...............E.....K.....................................................................................................
MSSA476     NC-002953.3   ...............E.....K.....................................................................................................
Newman      NC-009641.1   ....................................................................................................................................
SdrD1 strain nr.32-t084-CC84 ...NSKT.....................................................................K...............D.K..P.S.....QI.T...D
SdrD2 strain nr.19-t002-CC2  ...NSKT.....................................................................K...............D.K..P.S.....QI.T...D
SdrD3 strain nr.8-t024-CC24  ....................................................................................................................................
SdrD4 strain nr.46-t005-CC5  ...............A.....K.....................................................................................................
SdrD5 strain nr.10-t1102-CC78 ..............A.....................................................................................................
SdrD6 strain nr.43-t209-CC65 ...............T.....K.....................................................................................................
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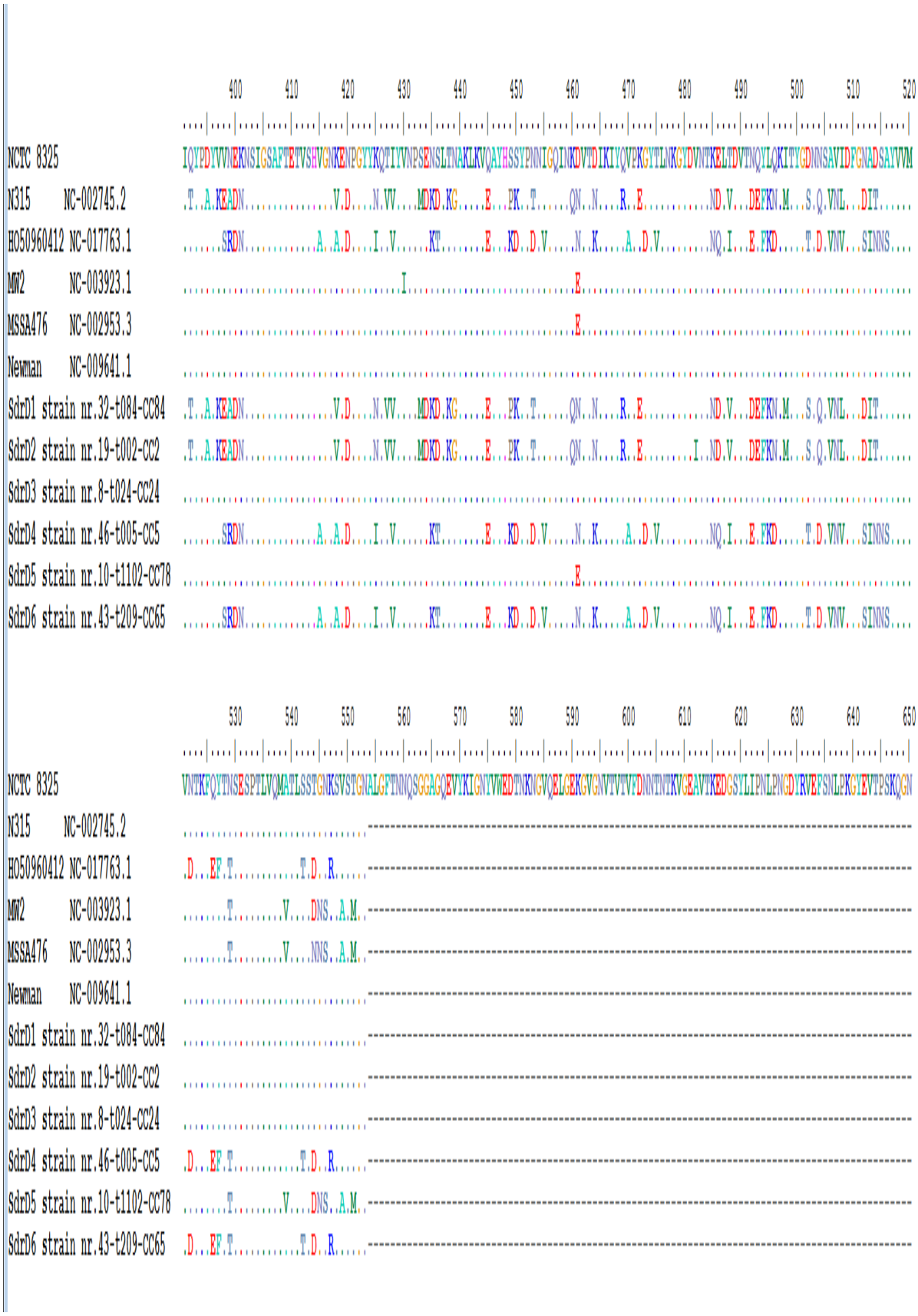
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N315 NC-002745.2
HO50960412 NC-017763.1
MW2 NC-003923.1
MSSA476 NC-002953.3
Newman NC-009641.1
SdrD1 strain nr.32-t084-cc84
SdrD2 strain nr.19-t002-cc2
SdrD3 strain nr.8-t024-cc24
SdrD4 strain nr.46-t005-cc5
SdrD5 strain nr.10-t1102-cc78
SdrD6 strain nr.43-t209-cc65
530 540 550 560 570 580 590 600 610 620 630 640 650
NCTC 8325
N315 NC-002745.2
HO50960412 NC-017763.1
MW2 NC-003923.1
MSSA476 NC-002953.3
Newman NC-009641.1
SdrD1 strain nr.32-t084-cc84
SdrD2 strain nr.19-t002-cc2
SdrD3 strain nr.8-t024-cc24
SdrD4 strain nr.46-t005-cc5
SdrD5 strain nr.10-t1102-cc78
SdrD6 strain nr.43-t209-cc65