Characterization of a Δ*nth* mutant in *Staphylococcus carnosus* TM300 and a Δ*PII-like* mutant in *Staphylococcus aureus*

Dissertation

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…dedicated to my family

CHAPTER I

Characterization of a Δ*nth* mutant and improvement of transformation efficiency in *Staphylococcus carnosus* TM300

Abbreviations

Am/Am^R	ampicillin/ampicillin resistance
AP	apurinic/apyrimidinic
BER	base excision repair
BM	basic medium
Bs	base pair(s)
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cfu	colony forming unit
Chisom	chlorophorm/isoamylalcohol
Cm/Cm^R	chloramphenicol/chloramphenicol resistance
dH_2O	distilled water
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
Em/Em^R	erythromycin/erythromycin resistance
ermB	erythromycine resistance cassette B
ERT	Eppendorf reaction tube
EtBr	ethidium bromide
EtOH	ethanol
Fig.	figure
h	hour(s)
HCl	hydrogen chloride
H_2O_2	hydrogen peroxide
ITC	Isothermal titration calorimetry
kb	kilobase
kDa	kilodalton
M	molar
mA	milli-ampere
MCS	multiple clonging site
MetOH	methanol
ml	milliliter
mM	millimolar
NaCl	sodium chloride
NaOH	sodium hydroxide

Nm	nanometer
nt	nucleotide
ONC	overnight culture
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethyleneglycol
REN	restriction endonuclease
rpm	round per minute
RT	room temperature
SD	Shine Dalgarno
SDS	sodium dodecylsulfate
Tab.	table
TAE	tris acetate EDTA
Tet/Tet^R	tetracycline/tetracycline resistance
UV	ultraviolet
V	volt
v/v	volume per volume
Vol.	volume
w/v	weight per volume

Amino acid code

A	Ala	alanine	I	Ile	isoleucine	R	Arg	arginine
C	Cys	cysteine	K	Lys	lysine	S	Ser	serine
D	Asp	aspartic acid	L	Leu	leucine	T	Thr	threonine
E	Glu	glutamic acid	M	Met	methionine	V	Val	valine
F	Phe	phenylalanine	N	Asn	asparagine	W	Trp	tryptophan
G	Gly	glycine	P	Pro	proline	Y	Tyr	tyrosin
H	His	histidine	Q	Gln	glutamine			

Symbols

(Δ) deletion	(~) circa	(λ) lambda	(°C) Grad Celsius	(::) insertion
(Φ) phi	(μ) micro	(Ψ) psi		

A. Abstracts

A.1 English abstract

DNA nucleases catalyze the cleavage of phosphodiester bonds. These enzymes play crucial roles in various DNA repair processes, which involve DNA replication, base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair. Some of the nucleases limit DNA uptake efficiency in bacteria by degrading foreign DNA. Rosenstein *et al.,* have annotated several genes within the genome of *Staphylococcus carnosus* TM300 with similarity to nucleases. Among these genes, there was one homologous to the putative endonuclease III gene, *nth*. Endonuclease III is described to cleave phosphodiester bonds at apurinic or apyrimidinic sites in DNA and has important functions in some steps during base-excision repair of DNA. Homologous *nth* endonucleases are found nearly in all organisms, including humans. In this study we aimed to generate an *nth* (*sca_1086*) deletion mutant in *S. carnosus* TM300 and investigate its function by comparative phenotypic analysis. To delete *nth*, a knock-out plasmid pBT2Δ*nth::erm*B was constructed including *lox* sites. Resistant colonies against erythromycin and chloramphenicol were screened and the resulting Δ*nth* mutant was verified by DNA sequencing. Subsequently an erythromycine resistance cassette (*erm*B) was excised from genomic DNA by *Cre* recombinase which is expressed from plasmid pRAB1. Wild type (WT) and Δ*nth* mutant strains have been characterized comparatively. Nuclease activity in cell crude extracts was assayed using undigested λ-DNA as a substrate. Indeed, in Δ*nth* mutant, degradation of λ-DNA was significantly decreased compared to WT strain. As *S. carnosus nth* might also be involved in DNA repair the susceptibility to various mutagens was investigated. Two differently acting mutagens were tested: hydrogen peroxide and mitomycin-C. H_2O_2 is not only a strong oxidizer, it is also a mutagen, which damages DNA, and mitomycin-C is a potent DNA-crosslinker and efficiently kills bacteria. While the minimal inhibition concentration (MIC) of mitomycin-C was unchanged, the survival rate of the Δ*nth* mutant to H_2O_2 or streptomycin stress was clearly impaired. The mutagenic survival assay was carried out by the classical Ames-test. It turned out that the frequency of spontaneous resistant mutants against streptomycin and mitomycin-C was approximately three-fold increased in Δ*nth* mutant, suggesting that the DNA repair activity was impaired. Finally, the transformation efficiency of Δ*nth* and WT strain was

investigated and it was found that Δ*nth* mutant showed a three-fold higher transformation efficiency compared to WT. These results show that the putative endonuclease III in *S. carnosus* TM300 has important functions in both, DNA repair and degradation of exogenic DNA.

A.2 Deutsche Zusammenfassung (German abstract)

DNA-Nukleasen sind Enzyme, die die Spaltung von Phosphodiester-Bindungen katalysieren. Sie spielen eine bedeutende Rolle in verschiedenen DNA-Reparatur-Prozessen, wie der Basen exzisionsreparatur, Nukleotid-exzisionsreparatur, Mismatch-Reparatur und Doppelstrangbruch- Reparatur sowie der DNA-Replikation. Einige dieser Nukleasen begrenzen die DNA- Aufnahmeeffizienz in Bakterien durch den Abbau der Fremd-DNA. Im Genom von *Staphylococcus carnosus* TM300 annotierten Rosenstein *et al.,* mehrere Gene mit Ähnlichkeiten zu Nukleasen. Unter diesen befindet sich ein zur Endonuklease III homologes Gen, welches in der *S. carnosus* Genomdatenbank die Bezeichnung sca_1086 trägt. Diese Endonuklease spaltet Phosphodiesterbindungen an apurinischen oder apyrimidinische Stellen in der DNA und spielt eine wichtige Rolle bei der DNA Basen exzision reparatur. Zu *nth* homologe Gene können in fast allen Organismen, einschließlich dem Menschen, gefunden werden. Das Ziel dieser Studie war es eine *nth* (sca_1086) Deletionsmutante in *S. carnosus* zu generieren, um die Funktion des *nth* Gens durch vergleichende phänotypische Analysen aufzuklären. Um das *nth* Gen deletieren zu können, wurde das geloxte Knock-out Plasmid pBT2Δ*nth*::*erm*B konstruiert. Durch ein Screening nach Erythromycin-resistenten und Chloramphenicol-sensitiven Klonen wurde eine Δ*nth* Deletionsmutante identifiziert, welche durch eine DNA-Sequenzierung verifiziert wurde. Danach wurde die Erythromycin-Resistenz-Kassette (*erm*B) aus der genomischen DNA durch eine Cre-Rekombinase ausgeschnitten, welche auf dem Plasmid pRAB1 codiert wird. Der Wildtyp (WT) und die erzeugten Δ*nth* Mutantenstämme wurden durch eine Reihe von phänotypischen Versuchen verglichen. Die Nuklease-Aktivitäten von Rohzellextrakten wurden unter Verwendung unverdauter λ-DNA als Substrat bestimmt. Tatsächlich war der Abbau von λ-DNA in der Δ*nth* Mutante im Vergleich zum Wildtyp signifikant verringert. Da das *S. carnosus nth* Gen an der DNA-Reparatur beteiligt sein könnte wurde auch die Anfälligkeit für verschiedene Mutagene untersucht. Zwei unterschiedlich wirkende Mutagene wurden getestet: Wasserstoffperoxid (H_2O_2) und Mitomycin-C. H_2O_2 ist nicht nur ein starkes Oxidationsmittel, es kann auch die DNA verändern und schädigen. Mitomycin-C ist ein potenter DNA-Crosslinker und tötet daher Bakterien effizient ab. Während die minimale Hemmkonzentration (MHK) von Mitomycin-C unverändert blieb, wurde die Überlebensrate der Δ*nth* Mutanten unter H_2O_2 oder Streptomycin Stress deutlich

verringert. Um die Anzahl an überlebenden Mutanten nach Zugabe der mutagenen Substanzen zu ermitteln, wurde ein klassicher Ames Test durchgeführt. Es stellte sich heraus, dass die Frequenz der spontanen Streptomycin- und Mitomycin-Resistenzen in der Δ*nth* Mutante etwa dreifach gesteigert wurde, was darauf hindeutet, dass die DNA-Reparatur beeinträchtigt wurde.

Zuletzt wurde die Transformationseffizienz der Δ*nth*-Mutante und des WT-Stammes untersucht und es konnte gezeigt werden, dass die Δ*nth*-Mutante eine dreifach erhöhte Transformationseffizienz im Vergleich zum WT besitzt. Diese Ergebnisse zeigen, dass *nth* sehr wahrscheinlich für eine Endonuklease III in *S. carnosus* TM300 kodiert und sowohl an der DNA-Reparatur als auch am Abbau von exogener DNA beteiligt ist.

B. Introduction

B.1 Staphylococci

Staphylococci are non-sporulating, gram-positive, facultatively anaerobic cocci with a size of 0.5-1.5 µm in diameter and divide in multiple planes to form irregular clumps of cells. They are resistant to drying and tolerate high concentrations of salt (10% NaCl) when grown on artificial media. Staphylococci are readily dispersed in dust particles through the air and on surfaces. Some species of this family are very important human pathogens such as *Staphylococcus epidermidis* (*S. epidermidis*), a non pigmented species usually found on the skin or mucous membranes and *Staphylococcus aureus* (*S. aureus*), a yellow pigmented species which more commonly associated with human disease. Both species are frequently present in the normal microbial flora of the upper respiratory tract and the skin (Madigan & Martinko, 2009).

B.1.1 *Staphylococcus carnosus* (*S. carnosus*)

Gram-positive and catalase-positive cocci have significant roles in the ripening process of dry sausages and meat. *S. carnosus* is one of the predominant microorganism in the fermented meat industry and this species was actually isolated from meat (Freudl, 2005). It was first classified as a new species in 1982 by Schleifer and Fischer (Götz *et al.*, 2006) and was grouped as a member of the genus *Staphylococcus*, based on several criteria such as DNA sequence homology, peptidoglycan composition and biochemical properties (Freudl, 2005). *S. carnosus* and *Staphylococcus xylosus* (*S. xylosus*) are non-pathogenic species within the genus *Staphylococcus*. *S. carnosus* produces none of the virulence factors associated with pathogenic staphylococci such as protein A, coagulase, hemolysins and toxins and exhibit a low homology to *S. aureus* (Götz, 1990). It is widely used in the food industry as a meat starter culture or in biotechnology as cloning host (Rosenstein *et al.*, 2009). In sausage ripening, the Gram-positive *S. carnosus* is inoculated as part of a starter culture in order to lower pH and to enhance the characteristic taste and aroma of cured sausages by the production of certain ketones (Fadda *et al.*, 2002). *S. carnosus* was firstly distinguished by its ability to grow in high NaCl concentrations (up to 15%) with a cell size around 0.5-1.5 µm in diameter and forms grey-white colonies. Also they reduces nitrate to nitrite, which in turn prevents bacterial growth of opportunistic species and in a reaction with

myoglobin produces a dark red color, generally considered more appetizing than grey. It is almost destitute of extracellular proteolytic activity and has thus been used quite widely for secreted production of recombinant proteins (Götz, 1990, Dilsen *et al.*).
S. carnosus TM300 was identified as a host for the expression, secretion and surface display of heterologous proteins. Introduction of DNA into *S. carnosus* was carried out by protoplast transformation (Götz *et al.*, 1983, Götz & Schumacher, 1987) or electroporation (Augustin & Götz, 1990, Löfblom *et al.*, 2007). In addition, a variety of vectors suitable for cloning and the expression of genes have been developed (Kreutz & Götz, 1984, Keller *et al.*, 1983, Wieland *et al.*, 1995). Moreover studies for gene replacement (Brückner, 1997, Madsen *et al.*, 2002) and promotor investigation (Wieland *et al.*, 1995) and for secretion (Liebl & Götz, 1986) have been demonstrated. Furthermore, a vector system has been defined for *S. carnosus*, which is based on the genes mediating sucrose uptake and hydrolysis in *S. xylosus* (Brückner & Götz, 1996).

B.2 Endonucleases

Replication, transcription and repair are very important and essential cellular metabolic processes for DNA. Some properties, principally during repair, need to be controlled by the hydrolysis of DNA. The enzymatic disruption of nucleic acids was firstly defined from Araki in 1903 and Iwanoff has been described in 1903 expression of '*nucleases'* for such enzymes. Nucleases can be further classified into exonucleases, which hydrolyse either from the 5′ or the 3′ end of nucleic acids, and endonucleases, which cleave internal phosphodiester bonds without the requirement of a free DNA end (Marti & Fleck, 2004).

B.2.1 Endonuclease III (*nth*)

(synonyms: DNA-(apurinic or apyrimidinic site) lyase; AP lyase)
Endonuclease III, was initially identified as an endonuclease which specifically cleaves DNA damaged by X-rays, UV light or free radicals (Gros *et al.*, 2002). Subsequently, it was revealed to be identical to endonuclease III, a repair enzyme recognizes a broad spectrum of oxidized pyrimidines (Katcher & Wallace, 1983). Endonuclease III is present in most cells and orthologs of *E. coli* endonuclease III have been cloned, overexpressed, and characterized including *Saccharomyces cerevisiae*, *Schizasaccharomyces pombe*, mice, humans, and many other organisms. The

enzyme was shown to have a DNA glycosylase activity with an associated AP (apurinic or apyrimidinic site) lyase enzyme activity which is able to nick DNA including an AP site by a β-elimination reaction (Kow, 2006). The nicking activity at abasic sites of *nth* is due to its AP lyase function. The phosphodiester bond cleavage occurs via β-elimination, generating 5' ends bearing 5'-phosphate and 3' ends with 2,3-unsaturated abasic residue 4-hydroxy-2-pentanal (Bailly, 1987). Most endonuclease III contains a [4Fe–4S] cluster which has been shown to be stable and resistant to both oxidation and reduction (Kuo *et al.*, 1992). Endonuclease III is initiating base-excision repair (BER) pathway at sites of DNA base damage and the lesion is processed by a DNA glycosylase. The downstream enzymes in the BER cascade complete the repair process by completely removing the processed lesion nucleoside, then repolymerizing and resealing the DNA backbone (Lindahl & Wood, 1999) (Fig. 1.).

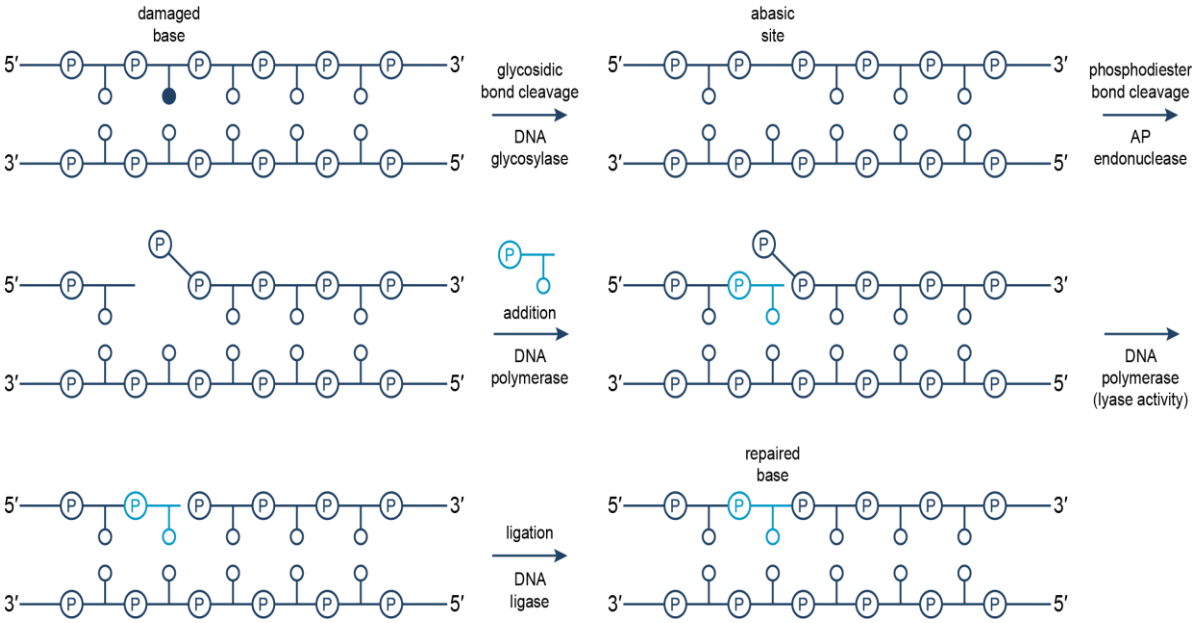


Figure 1. DNA base excision repair

A DNA glycosylase enzyme is recognized and excised the damaged base by leaving an abasic (AP) site. An AP endonuclease cleaves the phosphodiester bond at the 5′-end of the damaged base. DNA polymerases add a nucleotide monophosphate to replace the damaged base, which is then removed by the lyase activity of the polymerase. Finally, a DNA ligase closes the gap to complete DNA repair (Anonymous, 2012).

B.3 Transformation efficiency in prokaryotes

Transformation of foreign DNA into biological cells is an obligatory approach in biological science. Natural or artificial semi-permeability of bacterial cell wall enables the entry of exogenous DNA molecules into cell interior (Sambrook & Russell, 2001). Nevertheless, delivery of exogenous DNA is quite inefficient and in general even the best commercial competent cells only give transformation efficiencies of about 10^9–10^{10} cfu/µg plasmid DNA. Therefore, transformation is still an important obstacle to be overcome in some applications such as mutant library construction, establishment of mega plasmids in metagenomics and even
sometimes in regular cloning procedures (Aachmann & Aune, 2009). Consequently, improving the efficiency of transformation will result in both an economical and a time saving advantage for research areas of life science and biotechnological industries. In the 1970s, it could be shown that bacteria treated with ice-cold solutions of $CaCl_2$ followed by heat-shock could take up foreign DNA (Mandel & Higa, 1970, Cohen *et al.*, 1972). Many variations have been made to the basic technique described by including the addition of certain chemicals (Hanahan, 1983, Hanahan *et al.*, 1991, Panja *et al.*, 2006) and divalent cations (Norgard *et al.*, 1978) making transformation more effective. The structure of bacterial cell wall is more complicated than its mammalian counterpart, consisting of a multilayered barrier that functions to protect the bacterial cell from the surrounding environment (Aachmann & Aune, 2009). In general, Gram-positive bacteria are more resistant to plasmid DNA transformation than Gram-negative bacteria. This property is mainly referred to the structural differences in the cell wall barrier between this two cell types. The Gram-negative bacteria cell wall is composed as a dual membrane structure separated by a thin peptidoglycan layer. In contrast, the cell wall of Gram-positive bacteria is much thicker and consist with a single membrane and a massive outer peptidoglycan layer, acting as a physical barrier for the DNA, to obstruct efficient transformation of exogenous DNA (Löfblom *et al.*, 2007). Genetic transformation needs the development of efficiency, a genetically organized transient state permitting uptake of DNA. The introduction of foreign DNA into bacteria by means of transformation is a basic method in the genetic analysis of bacteria (Berge *et al.*, 2002). Thus, nucleases can be unfavorable, when genetic transformation techniques are used. The frequency of genetic transformation can decrease dramatically if the plasmid is degraded by nucleases (Ferreira, 1998). Mink *et al.* (1990) observed an

increase in transformation efficiency of *Saccharomyces cerevisiae* and *Aspergillus nidulans* when protoplasts were obtained with an endonuclease-free enzyme preparation (Mink *et al.*, 1990).

B.4 Aims of this study

Until today chemical, enzymological and crystallographic properties of staphylococccal nuclease (micrococcal nuclease) have been investigated (Tucker *et al.*, 1978, Tucker *et al.*, 1979). Additionally, expression and secretion of staphylococcal nucleases in other microorganisms (Pines & London, 1991, Liebl *et al.*, 1992) and genetic analysis have been studied (Shortle & Lin, 1985). Furthermore, mutagenesis and *in vitro* expression of thermostable nuclease in *S. aureus* has been analyzed (Tang *et al.*, 2008, Tang *et al.*, 2010). In this study, we aimed to investigate the functions of a putative endonuclease III in *S. carnosus* TM300 and show its roles on transformation efficiency.

For this purpose, main goals of this study were;

I. Creation of an endonuclease III (sca_1086; *nth*) deletion mutant in *S. carnosus* TM300 by homologous recombination
II. Comparative phenotypic characterization of deletion mutant with wild type strain
III. Improvement of transformation efficiency (to increase of foreign DNA uptake)

C. Materials and Methods

C.1 Materials

C.1.1 Chemicals

Tab. C.1 Used chemicals and their source of supply

Chemicals	Supplier
Acetic acid 100% p.a.	Carl Roth, Karlsruhe
Agar Kobe I	Carl Roth, Karlsruhe
Agarose peqGOLD Universal	PEQlab, Erlangen
Albumin from Bovine Serum (BSA)	Sigma-Aldr., Deisenhofen
Ampicillin Sodium salt	Carl Roth, Karlsruhe
Antibiotic Medium 3	Difco, Augsburg AppliChem, Darmstadt
Casein Enzymatic Hydrolysate, N-Z-Amine A	Sigma-Aldr., Deisenhofen
Chloramphenicol	Carl Roth, Karlsruhe
Chloroform	Carl Roth, Karlsruhe
D-(+)-Galactose	Carl Roth, Karlsruhe
D-(+)-Glucose	Carl Roth, Karlsruhe
D-(+)-Saccharose	Carl Roth, Karlsruhe
D-(+)-Xylose	Carl Roth, Karlsruhe
Dipotassium phosphate	AppliChem, Darmstadt
Disodiumhydrogenphosphat Dihydrate	Carl Roth, Karlsruhe
dNTP-Mix (10 mM each)	Fermentas, St. Leon-Rot
Erythromycin	Sigma-Aldr., Deisenhofen
Ethanol absolut p.a.	Carl Roth, Karlsruhe
Ethidiumbromid	Merck, Darmstadt
Ethylendiamintetraaceticacid (EDTA)	Carl Roth, Karlsruhe Fisher Scient., Schwerte
Glycerin	Carl Roth, Karlsruhe
Glycin	Carl Roth, Karlsruhe

Chemicals	**Supplier**
Hydrochloric acid 32%	Carl Roth, Karlsruhe
Isoamylalcohol	Merck, Darmstadt
Isopropanol (2-Propanol)	Carl Roth, Karlsruhe
Magnesium chlorid hexahydrate	Carl Roth, Karlsruhe
Maleic acid	Carl Roth, Karlsruhe
Methylenblue	Serva, Heidelberg
Mitomycine-C	Sigma-Ald., Deisenhofen
Oxytetracyclin	AppliChem, Darmstadt
Peptone from Casein, enzymatic digest	AppliChem, Darmstadt
Polyethylenglycol (PEG) 6000	Carl Roth, Karlsruhe
Potassium dihydrogen phosphate	Merck, Darmstadt
Saccharose	Carl Roth, Karlsruhe Serva, Heidelberg Sigma-Ald., Deisenhofen
Sodium Dodecylsulfat (SDS)	Merck, Darmstadt
Sodium succinate	Merck, Darmstadt
Sodiumchlorid	Carl Roth, Karlsruhe
Sodiumdihydrogenphosphat Dihydrat	Carl Roth, Karlsruhe
Sodiumhydroxide	Carl Roth, Karlsruhe
Soy Pepton	Carl Roth, Karlsruhe
Soy Peptone No. 110	Invitrogen, Karlsruhe
Streptomycin	Sigma-Ald., Deisenhofen
Tetracyclin	Sigma-Ald., Deisenhofen
Yeast extract Ohly Kat	D. Hefewerke, Nürnberg

C.1.2 Molecular biological Kits

Tab. C.2 Used molecular biological kits and their source of supply

Supplier	Kit
Fermentas GmbH, St. Leon-Rot	Rapid Ligation Kit CloneJet™ PCR cloning Kit
QIAGEN GmbH, Hilden	QIAquick-Gel extraction Kit, QIAGEN-Plasmid-Midi-Kit QIAGEN-Plasmid-Mini-Kit QIAquick PCR Purification Kit
Roche, Mannheim	Rapid DNA Dephos & Ligation Kit

C.1.3 Enzymes

Tab. C.3 Used enzymes and their source of supply

Supplier	Enzyme
Genaxxon BioScience GmbH, Ulm	ReproFast Polymerase
Genmedics GmbH, Reutlingen	Lysostaphin
Fermentas GmbH, St. Leon-Rot	restriction endonucleases, T4-DNA ligase
New England Biolabs, Schwalbach	restriction endonucleases
Fermentas GmbH, St. Leon-Rot	Shrimp Alkaline Phosphatase (SAP)

C.1.4 Molecular weight markers

Tab. C.4 Used molecular weight markers, loading dyes and their source of supply

Supplier	Product
Fermentas, St. Leon-Rot	GeneRulerTM 1 kb DNA ladder GeneRulerTM 1 kb plus DNA ladder
Qiagen, Hilden, Germany	Gel Pilot Loading Dye, 5X
Genaxxon BioScience GmbH, Ulm	undigested λ-DNA from *E. coli*

C.1.5 Consumable materials and plastic articles

Tab. C.5 Used consumable materials, plastic articles and their source of supply

Supplier	Product
Eppendorf, Hamburg	1.5 ml & 2 ml Eppendorf reaction tubes (ERTs)
GE Healthcare Europe, München	filter sterilizer (0.2 µm) (Whatman, Dassel)
Greiner Bio-One, Frickenhausen	tube PS 12 ml, Falcon™ tube PS 50 ml, single-use inoculating loop
Henke Sass Wolf, Tuttlingen	Injection Norm-Ject (1 ml, 5 ml, 20 ml)
Millipore, Schwalbach	Filter Millipak Express 20 (0.22 µm), Stericup™, PES membrane (0.22 µm)
Thermo Fisher Scientific	96 well plate Nunclon™ surface 96 well plate Nunc black U96 PP-0.5 ml

C.1.6 Computer programs

Tab. C.6 Used computer programs and their source of supply

Supplier / Source of Reference	Product
KEGG (http://www.genome.jp/kegg/)	Kyoto Encyc. of Genes and Genomes
NCBI, Bethesda (http://blast.ncbi.nlm.nih.gov/Blast.cgi)	Basic local alignment search tool (BLAST)
Ibis Therapeutics, Carlsbad	BioEdit 7.0.9.0
Invitrogen, Karlsruhe	VectorNTI Suite 10.3
Scientific-Educational Software, USA	Clone Manager Professional 9
DNASTAR, Inc., USA	SeqMan NGen
Microsoft Dtld. GmbH, Unterschleißheim	Microsoft Office 2010

C.1.7 Special machines used in the laboratory

Tab. C.7 Special machines and their source of supply

Supplier	Laboratory equipment
Beckman Instruments GmbH, München	Spectrophotometer DU 7500
Bio101 Thermo Savant	FastPrep FP120
Eppendorf AG, Hamburg	Centrifuge 5804R
Amersham Biosciences, Freiburg	ImageMaster®VDS (Gel documentation system)
Eppendorf, Hamburg	Pipettes
Heraeus GmbH, Osterode	Minifuge RF, Biofuge 13
IKA®-Werke GmbH & CO. KG, Staufen	IKA RCT IKAMAG magnet stirrer
Kern & Sohn GmbH, Balingen	Weighing machine, Kern EW 4200-2NM
Knick elektron Messgeräte, Berlin	pH meter
Leica Microsystems GmbH, Wetzlar	Leica Microscope Dm 5500 B +
Liebisch, Bielefeld	heating block
Millipore Corp., Billerica	Ultrapure water apparatus Milli-Q Biocel A10
MWG Biotech, Ebersberg	Primus & Primus 96 plus Thermocycler
New Brunswick Scientific, New Jersey	Innova 44 Shaking Series
Sartorius AG, Göttingen	special accuracy weighing machine, Sartorius BP61
Scientific industries, Inc., Bohemia	Vortex Genie 2
Sorvall, Bad Homburg	refrigerated centrifuges RC-5C and RC26 plus
Systec GmbH, Wettenberg	Autoclav Systec DX-150
Uniequip, Martinsried	Univapo 100 H (vacuum centrifuge)
UVP Inc., San Gabriel	Transilluminator
Biozym Scientific, Oldendorf	AlphaImager® Gel Documentation
Bio-Rad Laboratories, Munich, Germany	Electroporation apparatus Gene Pulser™ + Pulse Controller

C.1.8 Bacterial strains

Tab. C.8 Used bacterial strains

Strain/ Plasmid	Characteristics / Genotype	Source of Reference
Staphylococcus carnosus TM300	SK311	(Schleifer & Fischer, 1982)
Escherichia coli DH5α	*endA1 hsdR17* (rK- mK+) *supE44 thi-1recA1 gyrA* (Nalr) *relA1* Δ(*lacZYAargF*) *U169* *deoR* (Φ80 *dlacZ*ΔM15)	(Hanahan, 1983)
pBluescript® II	KS (+)	
Staphylococcus aureus RN4220	Derivative of NCTC8325-4. r- m-	(Herbert *et al.*, 2010) (Iordanescu & Surdeanu, 1976)

C.1.9 Plasmids

Tab. C.9 Plasmids

Plasmid	Size (kb)	Host	Selection	Source of Reference
pBT2	6.9	*E. coli* *S. c.* TM300	Am^R (100 µg/ml) Cm^R (10 µg/ml)	(Brückner, 1997)
pBT2_*tag*OermB	10.26	*E. coli*	Cm^R (10 µg/ml)	(Albrecht, 2010)
pRAB1	8.3	*E. coli* *S. c.* TM300	Cm^R (10 µg/ml) Am^R (100 µg/ml)	(Leibig *et al.*, 2008)
pJET1.2/blunt	2.97	unknown	Am^R (100 µg/ml)	Fermentas
pTX30	8.78	*S. c.* TM300	Tet^R (25 µg/ml)	(Strauss *et al.*, 1998)

Tab. C.10 Constructed plasmids

Plasmid	Size (kb)	Host	Selection	Source of Reference
pBT2_*nth*ermB	10.30	*E. coli* *S. c.* TM300	Am^R (100 µg/ml) Cm^R (10 µg/ml) Em^R (2,5 µg/ml)	(this work) (this work)
pTX30*nth*		*S. aureus* RN4220 *S. carnosus* TM300 Δ*nth*	Tet^R (25 µg/ml) Tet^R (25 µg/ml) Tet^R (25 µg/ml)	(this work) (this work) (this work)

C.1.10 Oligonucleotides

Synthetic oligonucleotides were ordered through Biomers (Ulm, Germany).

Tab. C.11 Oligonucleotides for cloning, PCR identification and verification

Primer	**Sequence (5'→3')**	**REN**	**Purpose of use**
Fwd_*nth*L_*Eco*RI	TTATAGAATTCACATATCAACAATACAACTTACCTGATG	*Eco*RI	Δ*nth*-upstream
Rev_*nth*L_*Sma*I	TAATACCCGGGGGACTAAAAGTCATCATCAACTC	*Sma*I	Δ*nth*-upstream
Fwd_*nth*R_*Sma*I	TATTACCCGGGTTTATTTACTAGTACATCCGTACATTGC	*Sma*I	Δ*nth*-downstream
Rev_*nth*R_*Bam*HI	TAATAGGATCCATGGATTTCTGGTGTTGAACATG	*Bam*HI	Δ*nth*-downstream
*nth*_downstream_*Xma*I	TATACCCGGGCCTTCTTGTGAAGTCAGC	*Xma*I	complementation
*nth*_upstream_*Bam*HI	TTATGGATCCATACGCCACAAATGAAGC	*Bam*HI	complementation

Tab. C.12 Oligonucleotides for DNA sequencing and verification

Primer	**Sequence (5'→3')**
pBT2a	CCAGGCGTTTAAGGGCACCA
pBT2b	CACGGTGCCTGACTGCGTTAGCA
S1	AGTTGATCAAGGTATTCCTCTCG
S2	CGATCGATGTATTTGAAATTAATTTT
Fwd_pJET1.2	CGACTCACTATAGGGAGAGCGGC
Rev_pJET1.2	AAGAACATCGATTTTCCATGGCAG
Δ*nth* _seq primer_1	GTACTGTAAATGGTGGCAG
Δ*nth* _seq primer_2	CGGTACAATCGGATAACTG
Δ*nth* _seq primer_3	CATCTGGAAACATATCAGC
Δ*nth* _seq primer_4	CAAGCGTTGAATAACTCC
Δ*nth* _seq primer_5	CTTTGGTCGCCCGCTTTCG
Δ*nth* _seq primer_6	CGAATGCAGAGAAGGGC
Δ*nth* _seq primer_7	TTAATTCTAGCGACTGCAC
Fwd_*nth*L_*EcoR*I	TTATAGAATTCACATATCAACAATACAACTTACCTGATG
Rev_*nth*R_*Bam*HI RC	TAATAGGATCCATGGATTTCTGGTGTTGAACATG
pCX_KX_TX_seq_1	TCTTCCTTATTTGAGTGGG
*erm*_up	GAATTCCAGCTGGGTACCCGGG

C.1.11 Media for cultivation

The culture media were autoclaved for 20 min at 121 °C and 2 bar. For the preparation of plates 15 g agar were added per liter.

Tab. C.13 Media for cultivation

Culture medium	Composition
Basic medium	10 g casein hydrolysate, 5 g yeast extract, 5 g NaCl, 1 g K_2HPO_4, 1 g glucose, with dH_2O ad 1L, pH 7,2
B2-Medium	Casein Hydrolysat (or Soy Pepton) 10 g, Yeast extract 25 g, NaCl 25 g, K_2HPO_4 1 g, Glucose 5 g, dH_2O ad 1 L pH 7,5
SMMP medium	composed of 16 parts 2x SMM (1M sucrose, 40 mM $MgCl_2$, 40 mM maleic acid, pH 6.8; filter sterilized), 4xPAB (70 g/L Difco antibiotic medium 3; 12 min. autoclaved), 1 part 5% BSA solution (bovine serum albumin broth, filter sterilized)
DM3-agar	200 ml 5% agar, 500 ml 1M Na-succinate (pH 7.3 with succinic acid), 150 ml 3.3% casein hydrolysate/ 3.3% yeast extract, 100 ml potassium phosphate buffer (3.5% K_2HPO_4 /1.5% KH_2PO_4), 20 ml 1 M $MgCl_2$, 10 ml 50% glucose, 10 ml 5% BSA (dissolved in 2x SMM). The components were separately sterilized and combined at 55 °C
CY3-agar	composed at 45 °C of 25 parts CY-agar (2.5% soy peptone, 2.5% yeast extract, 1.5% NaCl, 1% agar, 25 parts 1 M Na succinate (pH 7.3 with succinic acid), 1 part Mix-solution (4 parts 1.5 M Na-β-glycerol phosphate, 2 parts 1 M $MgCl_2$, 1 part 5% BSA, 1 part 50% glucose
10x salt solution	4.066 g $MgCl_2*6H_2O$, 1.029 g $CaCl_2*6H_2O$, 0.1 g $MnCl_2*4H_2O$, 1.36 g $ZnCl_2$ with Milli-Q water add 1 L, filter sterilized

C.1.12 Antibiotics and media supplements

Tab. C.14 Antibiotics and media supplements

Supplement	Stock solution	Final concentration	Solvent
Ampicillin (Amp)	100 mg/ml	100 µg/ml	ddH_2O
Chloramphenicol (Cm)	10 mg/ml	10 µg/ml	70% EtOH
Erythromycin (Er)	10 mg/ml	2,5 µg/ml	70% EtOH
Tetracyclin (Tet)	10 mg/ml	25 µg/ml	70% EtOH
Mitomycin C (MitC)	0,5 mg/ml	1 µg/ml	ddH_2O
Streptomycin (Str)	10 mg/ml	1 µg/ml	ddH_2O
Glucose (Glc)	50% (w/v)	0,5%	ddH_2O
Magnesiumchlorid ($MgCl_2$)	1 M	-	ddH_2O
Xylose (Xyl)	25% (w/v)	0,5%	ddH_2O

All solutions were filter sterilized and stored at -20 °C. In terms of thermostability autoclaved media were complemented at < 50 °C with its respective antibiotic.

C.2 Methods

C.2.1 Cultivation and storage of bacteria

Cultures of staphylococci were grown in Basic (B)-medium on a shaker at 37 °C and 150 rpm. Cells were cultivated without glucose (catabolite repression), when a xylose inducible expression vector was used. Usually, cells were grown until an OD_{578} = 0.6 and the gene expression was induced by adding 0.5% xylose. *E. coli* strains were cultivated in B-medium, too. Bacterial growth was observed at an optical density OD_{578} nm with a spectrophotometer. The bacterial strains on plates were stored at 4 °C. In general, storage of *E. coli* was for 2-3 weeks and *S. carnosus* TM300 was for 6-8 weeks. For long-term storage of bacteria one volume of overnight culture was mixed with one volume of autoclaved freeze-medium (65% glycerol, 0.1 M $MgSO_4$, 25 mM Tris/HCl, pH 8.0) and stored at -80 °C.

C.2.2 Methods for working with nucleic acids

Standard methods were performed for DNA isolation and manipulation as described in Qiagen manuals. Solutions and buffers were assembled using fine chemicals and deionized purified Milli-Q water. The DNA was kept at 4 °C for short-term storage and at -20 °C for long term storage. Phenol/chloroform extraction and ethanol precipitation were used for purification and concentration of DNA.

C.2.2.1 Agarose gel electrophoresis of DNA

Different sizes of DNA fragments were separated by electrophoresis in TAE-agarose gels (14x 11 cm, 0.8 - 1.0% agarose in TAE buffer) at 120 V. DNA was mixed with 6x-DNA loading dye (5:1), before the gel run. The DNA-standards GeneRuler 1 kb DNA ladder or DNA ladder mix (Fermentas, St. Leon-Rot) were used as markers. DNA of analytical gels was stained in an EtBr staining solution (2 µg/ml) for 15 min. and decolorized in water for 15 min. Destained DNA was photographed under the UV-light. Preparative gels were stained in methylene blue (0.1% in dH_2O) for 5-10 min. and destained for 30-60 minutes in water. DNA bands were excised from the agarose gel using a scalpel, when became visible.

C.2.2.2 Isolation of plasmid-DNA from *E. coli*

Mini-preps employing alkaline lysis (Birnboim & Doly, 1979) were used to isolate recombinant plasmids from *E. coli* for routine screening. 5 ml B-medium was inoculated with a single colony and grown overnight at 37°C, 150 rpm. 2 ml of this culture was harvested by centrifugation (2,000×g, 4°C, 10 min) and resuspended in 250 µl solution P1 by vortexing. The suspension was mixed with 250 µl solution P2 by inversion and incubated at RT for 5 min and then 250 µl solution P3 was added and incubated on ice for 10 min. After centrifugation for 30 min (20,000×g, 4°C), the supernatant was transferred into a fresh eppendorf tube. DNA was precipitated by addition of equal volume of isopropanol and centrifugation (20,000×g, 4°C, 30 min). DNA pellet was washed once with 500 µl 70% ethanol, air dried and resuspended in 40 µl TE buffer or in Milli-Q water.

C.2.2.3 Isolation of plasmid-DNA from Staphylococcus strains

The isolation of plasmid-DNA were performed using the "QIAGEN Plasmid-Midi-Kits" (QIAGEN GmbH, Hilden). First, cells from a 50-100 ml overnight culture were centrifuged (4700 rpm, 10 min, 4 °C) and then pellets were resuspended in P1 buffer (up to 100 ml culture in 4 ml buffer). Per 4 ml buffer 30-45 µl lysostaphin (0.5 mg/ml) were added. The cell solution was incubated in incubator for 20 min at 37 °C for cell wall degradation. The additional steps were carried out according to the manufacturer's protocol.

C.2.2.4 Isolation of chromosomal DNA from *Staphylococcus carnosus* TM300

The staphylococcal chromosomal DNA was isolated using a modified version of the method from Marmur (Marmur, 1961). For this purpose, the cells from 10 ml of an overnight culture were sedimented by centrifugation and resuspended in 2 ml of buffer P1.

To lyse staphylococcal cell wall, 50 µl lysostaphin (0.5 mg/ml) was added and incubated for 30-45 min at 37 °C. The lysis of the cells was performed by adding 100 µl SDS solution (saturated in 45% ethanol) and incubation at 37 ° C for 5 min. Proteins were precipitated by the addition of 650 µl of a 5 M $NaClO_4$ solution. The overall approach was mixed with 3 ml Chisom (Chlorophorm/Isoamylalcohol), well mixed and then centrifuged for phase separation (4700 rpm, 10 min, RT). After the transfer of the upper aqueous phase into a new tube Chisom treatment was repeated, by followed centrifugation 2-3 times until the supernatant was clear. Afterwards, 6 ml of ethanol was added in a new tube and the DNA precipitated visible as white strands. DNA was fished briefly by a Pasteur pipette, swirled in 70% ethanol and 1 h dried in oven or at RT. Finally, chromosomal DNA was solved in 400 µl TE buffer or ddH_2O.

C.2.2.5 Isolation of DNA from agarose gels

DNA fragments were excised from agarose gels, using a scalpel and collected in a 2 ml Eppendorf reaction tube. The DNA was recovered using the "QIAquick Gel Extraction-Kit" (QIAGEN GmbH, Hilden). Recovery of DNA was carried out according to the manufacturer's protocol.

C.2.2.6 Cleavage, ligation and dephosphorylation of DNA

DNA cleavage with restriction endonucleases (RE) was carried out according to the manufacturer‘s protocol. For the analytical DNA cleavage 0.05-0.5 µg of DNA were applied, and for preparative use 0.5-5 µg of DNA was sufficient. The minimum and maximum reaction volume was set to 20 µl and 60 µl, respectively. All reactions with RE were carried out at 37 °C, except *Sma*I (30 °C). For the analytical purpose a reaction time of 1.5 hours and for the preparative usage a reaction time of 2.5-3 hours were applied. The RE with its appropriate buffers were obtained from New England Biolabs GmbH (Schwalbach) and Fermentas GmbH (St. Leon-Rot).
The ligation of free DNA ends was carried out with T4-DNA ligase (Fermentas, St. Leon-Rot). Therefore, DNA was prepared using preparative agarose gel electrophoresis. The vector insert ratio was 1:5. The ligation was carried out with the 10x ligation buffer and 1 µl ligase at 16 °C
or room temperature minimum 3 hours or overnight. The 5’-phosphate group of the free DNA-ends was enzymatically removed by Shrimp Alkaline Phosphatase (SAP), after the hydrolytic cleavage of a vector by a RE. Per µg DNA 1-10 U of SAP were added, and the reaction batch was incubated for 1h at 37 °C. Later, the SAP was heat-inactivated for 15 min. at 65 °C.

C.2.2.7 Polymerase chain reaction

To amplify specific DNA fragments *in vitro* (Mullis & Faloona, 1987, Bej *et al.*, 1991), we performed the polymerase chain reaction (PCR). Synthetic oligonucleotides (primer) complementary to the target sequence and flanking the to-be-amplifying region are used as start points for a thermostable DNA polymerase. Primer ends were often modified to include recognition sites for RE or an optimized ribosomal binding site. The basis of this technique is a DNA-Polymerase, of which the optimized temperature is *ca*. 75 °C. In general, the Reprofast polymerase (Genaxxon, Ulm) was used for analytical and colony PCRs. Common PCR was carried out in 50 µl reaction volume with 1x reaction buffer containing $MgCl_2$, 25 µM dNTP mix (dATP, dCTP, dGTP, dTTP), 0.5 µl primer (100 pmol/µl), 2 U polymerase and minimum 100 ng DNA template or 1-2 µl chromosomal DNA. Afterwards, 10% of the PCR-reaction was analyzed on an analytical agarose gel. A standard PCR-protocol is given in Tab. C.15.

Tab. C.15 Standard protocol for PCR

	Denaturation	***Annealing***	***Elongation***
Start	94 °C / 5.00 min.	-	-
Cycle 1-32	94 °C / 0.30 min.	estimated/ 1.30 min.	72 °C / estimated
End (cycle 33)	94 °C / 0.30 min.	estimated/ 1.30 min.	72 °C / estimated
Store at 8 °C			

C.2.2.7.1 Colony PCR

Colony-PCR is a fast analytical method to screen for positive clones after transformation of plasmid DNA. In this approach the whole bacterial colony was taken and its DNA served as a template in a PCR reaction instead of a purified DNA template. The analytical PCR was carried out in 20 µl total reaction volume via the Reprofast DNA polymerase for 20-25 cycles. A single colony was picked with a steril pipette tip or a sterile toothpick and transferred firstly into the prepared PCR-reaction solution and afterwards to a freshly prepared solid BM-agar plate complemented with the respective antibiotic. Afterwards, 5-10 µl of the PCR reaction were analyzed on an analytical agarose gel.

C.2.2.8 Purification of DNA products amplified by PCR

Two methods for the purification of the PCR products were used. Either the DNA was purified according to the protocol of the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden), when the PCR products were pure and visible as single bands on the analytical agarose gel, or the particular DNA fragment was cutted out of a methylene blue stained preparative agarose gel and further purified according to the protocol of the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden).

C.2.2.9 Sequence verification by DNA sequencing

Samples were sent to the sequencing company GATC in Konstanz. Therefore, PCR products of the to-be-sequenced DNA region were amplified from genomic DNA by Reprofast DNA polymerase (Genaxxon) or isolated-purified plasmid DNA was sent directly. In general, DNA sequences were analyzed by SeqMan NGen **(**DNASTAR, Inc., USA), BioEdit (Ibis Therapeutics, Carlsbad) and the Clone Manager Professional 9 (Scientific-Educational Software, USA). The BLAST program was used for the

comparison of DNA and amino acid sequences, over the internet at the “National Center for Biotechnology Information“ (NCBI, Bethesda, USA) (Altschul *et al.*, 1990, Altschul *et al.*, 1997).

C.2.3 Transformation of bacteria

C.2.3.1 Preparation of competent *E. coli* DH5α cells

To preparing of competent cells, *E. coli* DH5α was inoculated in 40 ml B-medium with 1:200 dilution of overnight culture. Cells were grown until an OD_{578} = 0.4 was reached. Afterwards, they were cooled down on ice for 10 min. before harvesting by centrifugation at 5’000 rpm for 10 min. at 4 °C. The cell pellet was resuspended in 20 ml ice-cold 50 mM $CaCl_2$ and was incubated for 25 min. on ice. Afterwards, cells were spinned down by centrifugation at 5’000 rpm for 10 min. at 4 °C. The cell pellet was resuspended in the heat shock buffer (2 ml of 50 mM $CaCl_2$ and 500 µl 50% glycerol), separated into aliquots of 100 µl each, and stored at -20 °C (Dagert & Ehrlich, 1979).

C.2.3.2 Transformation of competent *E. coli* DH5α by heat shock

Before transformation steps, competent cells were thawed on ice for 5 min. and 10 µl ligated plasmid was added. The cell solution was incubated at 42 °C for 90 sec in a heat block and afterwards it was transferred directly into ice. Subsequently, 1 ml B-medium was added and the cell solution was incubated for 1-1.5 hours shaking at 37 °C. Afterwards, 100 µl cell cultures were spread each on two BM agar plates, complemented with the respective antibiotics, and incubated overnight at 37 °C.

C.2.3.3 Transformation of *S. carnosus* by protoplast transformation

(Götz *et al.*, 1983, Götz & Schumacher, 1987)

C.2.3.4 Preparation of protoplasts

300 ml B-medium (1L flask with baffle) was inoculated with 1 ml ONC of *S. carnosus* TM300. Cells were grown shaking at 37 °C until OD_{578} = 0.35-0.45. After cooling down on ice (10 min.), cells were transferred to sterile centrifuge tubes and spinned down for 20 min. by centrifugation at 4500 rpm and 4 °C. The sedimented cells were

resuspended in 30 ml $SMMP_{75}$ medium and transferred to sterile 50 ml falcon tubes. Afterwards, 10 µl of a sterile filtered lysostaphin solution (0.5 mg/ml) was added, and the whole mixture was incubated statically overnight for 12-16 hours at 30 °C. The formation of protoplasts was monitored by light microscopy (Leica DME). The lysis process was stopped, when the cells were singularized and spheroplasts became visible. Subsequently, the protoplasts were spinned down by centrifugation for 20 min. at 4'500 rpm and room temperature (RT). After discarding the supernatant, residues of lysostaphin were removed by washing with 5 ml $SMMP_{75}$ medium. Carefully, the protoplast pellet was resuspended with 2 ml $SMMP_{75}$ medium and aliquoted to 300 µl portions in 12 ml Greiner tubes. The protoplasts were stored at -70 °C until use.

C.2.3.5 Transformation of *S. carnosus* TM300 protoplasts

Protoplasts of 300 µl were slowly thawed on ice (10 min.). The DNA (5-70 µl, 0.5-1.0 µg) was pipetted on the edge of the 12 ml Greiner tube, embedded by 2 ml Fusogen (40 g polyethylene glycol 6000, 50 ml 2x SMM, filled up with 100 ml dH_2O; filter-sterilized), and flushed into the protoplast solution. During the transformation process, the tube was carefully rotated and inverted for exactly 2 min. (PEG 6000 can damage the protoplasts while extending the transformation time). Addition of 7 ml $SMMP_{75}$ medium stopped the transformation.

Cells were spinned down for 20 min. by centrifugation (4'500 rpm, RT). The supernatant was discarded and the protoplast sediment was resuspended in the medium reflux and plated each on two DM-3 agar plates (Chang & Cohen, 1979, Götz & Schumacher, 1987). These were incubated for 3-4.5 hours at 37 °C for the regeneration of cell walls. Afterwards, 3 ml soft agar (CY3-soft agar/ Na-succinate solution/ Mix-solution, at a ratio of 5:5:1), including the respective antibiotics in a 10 fold concentration, were poured on the preincubated DM-3 agar-plates. These were incubated until colonies were formed, for 2-4 days at 37 °C (according to plasmid!).

C.2.3.6 Transformation of *S. carnosus* by electroporation

(Löfblom *et al.*, 2007)

Preparation of electrocompetent cells:

An overnight culture of *S. carnosus* cells was diluted into 500 ml B2 medium to an optical density at 578 nm (OD_{578}) of 0.5 and grown at 37 °C and 110 rev/min to an

OD_{578} of 0.6. The cells were incubated on ice for 15 min to stop growth, harvested by centrifugation (3000 g, 10 min and 4 °C), and washed with 500, 250 and 80 ml of ice-cold sterile water. Subsequent washing steps were performed with 10 and 5 ml ice-cold 10% glycerol. The cell pellet was resuspended in 2 ml of ice-cold 10% glycerol to a final concentration of around 4 $x10^{10}$ cells/ml. Finally, 60 µl aliquots of cells were immediately transferred for storage to a -80 °C freezer.

Electroporation procedure:

Electrocompetent *S. carnosus* cells were thawed on ice for 5 min, and incubated at room temperature for 30 min. The plasmid was thawed and 1 µl (4 µg/µl) was added to the cells, and the mixture was incubated at room temperature for 10 min prior to electroporation. After incubation with DNA, 50 µl of the cells were transferred to a 1-mm gap electroporation cuvette and transformed with an electroporation apparatus (Gene Pulser™ Bio-Rad Laboratories, Munich, Germany), using a field strength of 21 kV cm^{-1} and a pulse width of 1.1 ms. The complete electroporation procedure was performed at room temperature. Immediately after electroporation, 1 ml B2 medium was added to the cuvette, and the cells were transferred to a 14 ml tube and incubated at 37 °C and 130 rev/min for 2 h. Finally, the cells were diluted 1/200 in B2 medium, spread on BM agar plates with the respective antibiotics and incubated at 37 °C for 48 h.

C.2.3.7 Transformation of *S. aureus* by electroporation

Preparation of electrocompetent S. aureus cells:

Electrocompetent *S. aureus* RN4220 cells were prepared by a glycerol treatment. Briefly, 100 ml BM-media was inoculated with 1 ml of an overnight culture and grown at 37°C for 90 - 120 min. Upon reaching an OD_{578} of 0.5, the cells where centrifuged for 15 min at 4000 rpm and 4°C. The pellet was washed with 100 ml, 50 ml, and 25 ml 10% ice cold sterile glycerol, resuspended in 400 µl 10% cold glycerol and stored in 75 µl aliquots at 70°C.

Electroporation procedure:

Electrocompetent *S. aureus* RN4220 cells were thawed on ice, 1 µg (1-5 µl) of plasmid/ligation reaction was added and the mix was incubated for 15 min at room temperature. The competent cells were then transferred to a sterile 0.2 cm electroporation cuvette and electroporation was carried out with 2 kV (2.5 µF capacity,

100 Ω resistance), yielding time constants from 2.4 - 2.5 ms. Immediately after electroporation, 950 µl BM-medium was added to the cuvette, the suspension was transferred to a 2 ml reaction tube and incubated shaking at 37 °C for 90 min. 100 µl of the suspension was spread on B-medium solid media containing the necessary antibiotic. The residual suspension was concentrated in 100 µl backflow after 1 min of centrifugation and plated on a separate BM agar plate as before.

C.2.4 Knock-out of chromosomal genes in *S. carnosus* TM300

The method is based on the exchange of genes in the genome of *S. carnosus* using *homologous recombination* method. Herewith, the chromosomal encoded gene of interest can be exchange by an antibiotic resistance marker that also is called “gene knock-out“. Therefore, the shuttle vector pBT2 was used, which has a temperature sensitive replicon for staphylococci. No plasmid replication takes place, when cultivated above 30 °C. As selection marker, the erythromycin resistance cassette derived from plasmid pEC2 was used and the inactivation vector was cloned in *E. coli* (Brückner, 1997). Therefore, flanking regions of the target sequence were cloned into pBT2 (optimal size: each >1kb length) and the *to be deleted* region was replaced by the resistance cassette. The constructed plasmid was verified by sequencing, and inserted into *S. carnosus* TM300 by protoplast transformation. The selection occurred by the vector mediated resistance to chloramphenicol (Cm10) on BM agar plates. Plasmid DNA was isolated from *S. carnosus* TM300 and the inserted genes were controlled for failures by sequencing. Subsequently, an overnight culture was inoculated (50 ml) and cells were grown at 30 °C for the plasmid replication in the presence of Cm10.

Afterwards, 50 ml pre-heated medium (2.5 µg/ml Em) were inoculated with overnight culture (1:1000) and incubated at 42 °C in a water bath overnight. Now, the cells were cured from the pBT2 knock out plasmid, as its replication stopped at 42 °C and, consequently, the plasmid was diluted. A 50 ml preheated medium without antibiotics was inoculated with a 1:1000 dilution of an overnight culture and incubated overnight at 40 °C. From a dilution series ($1x10^{-5}$ - $1x10^{-6}$) overnight grown *S. carnosus* were picked on Em2.5 and Cm10 agar plates and incubated overnight at 37 °C. *S. carnosus* clones growing on Em 2.5 instead of Cm10 lost their plasmid, however, integrated the

resistance cassette into the chromosome. Contrary, *S. carnosus* colonies, which were able to grow on Em2.5 and Cm10 still carried the plasmid.

C.2.5 Removal of the resistance marker

Once an antibiotic resistance marker is integrated into the genome, it can not be reused for another gene knock out in the established method. Therefore, the erythromycin resistance cassette *erm*B was flanked by the so-called *lox* (locus of x-over P1)-sites, *lox66* and *lox71*. Subsequently, when the integrated antibiotic cassette was confirmed by sequencing, it was excised using *Cre*-recombinase, which specifically recognizes *lox66* and *lox71* and removes the space in-between, generating a *lox72*-site (Leibig *et al.*, 2008). The latter is poorly recognized by the *Cre*-recombinase. Consequently, another gene knock out could be carried out in this strain with an *erm*B cassette, which might be used again with *lox*-sites.

C.2.6 Degradation of undigested λ-DNA with crude extract of Δ*nth* mutant

Undigested λ-DNA from *E. coli* was used as a substrate for endonuclease assay. For this purpose strains of WT and Δ*nth* mutant were inoculated in 50 ml BM and incubated at 37 ºC overnight. Δ*nth*/pTX30*nth* was inoculated in BM without glucose. Additionally, 0.5% xylose and tetracycline (25 µg/ml) were added for the expression of *nth* gene. Next day, cultures were centrifuged in 50 ml Falcon tubes at 4700 rpm, 10 min at +4 ºC. Pellets of cultures were washed twice with 15 ml BM and centrifuged again at 4700 rpm, 10 min at +4ºC. Pellets were resuspended again in 15 ml BM. For the prevention of loss of enzyme activity, cells were kept on ice. Then, cells were sonicated (Sonifier 250/Branson; 3 min., setting 60% and stage 7) to get the cytoplasmic contents. After sonication, cell suspensions were centrifuged 10 min at 4700 rpm, +4 ºC and supernatants transferred in new Falcon tubes. Enzymatic reaction was performed in Eppendorf reactions tubes. 10 µl undigested λ-DNA (1:5 diluted from stock 300 µg/ml) and 490 µl from crude extracts (culture supernatants) were mixed and incubated at different time points (10 min and 30 min) at 37 ºC. Reactions were inactivated for 10 min at 70 ºC and placed at +4ºC. A subsequent ethanol precipitation was made and samples were solved in 100 µl MQ. After ethanol precipitation all samples were loaded on a 1% agarose gel.

C.2.7 H_2O_2 sensitivity test

The sensitivity test against hydrogen peroxide (H_2O_2) was performed according (Collier *et al.*, 2012). Wild type, Δ*nth* and Δ*nth*/pTX30*nth* strains were grown from fresh single colonies in BM at 37 °C to an OD_{600} of 0.12 and 0.14, respectively. Samples (1 ml) were removed and H_2O_2 was added (22, 45, 60, 90 and 120 mM). Distilled water instead of H_2O_2 was added to the control samples. All samples were incubated for 30 min at 37 °C with vigorous shaking, before serial dilutions (10^{-5}) were spotted on BM plates and left to grow at 37 °C overnight.

C.2.8 Determination of minimum inhibitory concentration (MIC) by microdilution

The MICs of streptomycin and mitomycin-C against WT, Δ*nth* and Δ*nth*/pTX30*nth* were determined by the microdilution method, using tryptic soy broth (TSB) and following the method described by Deutsches Institut für Normung (DIN, 2004). Streptomycin and mitomycin-C were diluted in a 96-well microtiter plate to final concentrations of 1.7 and 1.4 µg/ml, respectively. A 100 µl aliquotes of the bacterial suspension (to 10^{-11} *cfu*/ml) was inoculated and incubated at 37°C for 12 h. The MIC was determined as the lowest concentration of the respective substance at which the OD_{578}, did not exceed 0.1. All assays were performed three times.

C.2.9 Analysis of spontaneous mutation frequency of Δ*nth* mutant and susceptibility

The determined MICs of the mutagenic antibiotics streptomycin and mitomycin-C were used to generate spontaneous mutation in WT, Δ*nth* and Δ*nth*/pTX30*nth* cells. As a first step, the strains were inoculated in TSB for overnight. Δ*nth*/pTX30*nth* was inoculated in TSB prepared without glucose and with 0.5% xylose and 25 µg/ml tetracycline. Next day, fresh inoculums were prepared from overnight cultures and incubated until an OD_{578} 0.5. Then 1.55 – 1.7 µg/ml streptomycin and 1.4 µg/ml mitomycin-C were added into cultures and incubated for 12 h. After 12 h, cultures were diluted in saline solution until 10^{-11} and 100 µl from the dilutions 10^{-9}, 10^{-10} and 10^{-11} were spread on BM agar plates with antibiotics. The number of spontaneous mutant colonies (*cfu*) were counted and compared.

D. RESULTS

D.1 Inactivation of *nth* in *S. carnosus* TM300 and complementation

In *S. carnosus* TM300, *nth* is located in the middle of an operon with a 672 bp nucleotide size. To generate a *nth* deletion mutant, an allelic replacement shuttle vector pBT2Δ*nthermB* (Fig. 2.), which contained an expression cassette of the erythromycin resistance gene (*erm*B) flanked by two DNA fragments from the flanking region of the *nth* gene, was designated and transformed into *S. carnosus* TM300 by electroporation or protoplast transformation. Approximately 200 erythromycin- and chloramphenicol-resistant colonies were selected, in which the existence of the allelic replacement vector pBT2Δ*nth* was confirmed by plasmid reisolation, *erm*B-PCR amplification, control digestions and by sequencing.

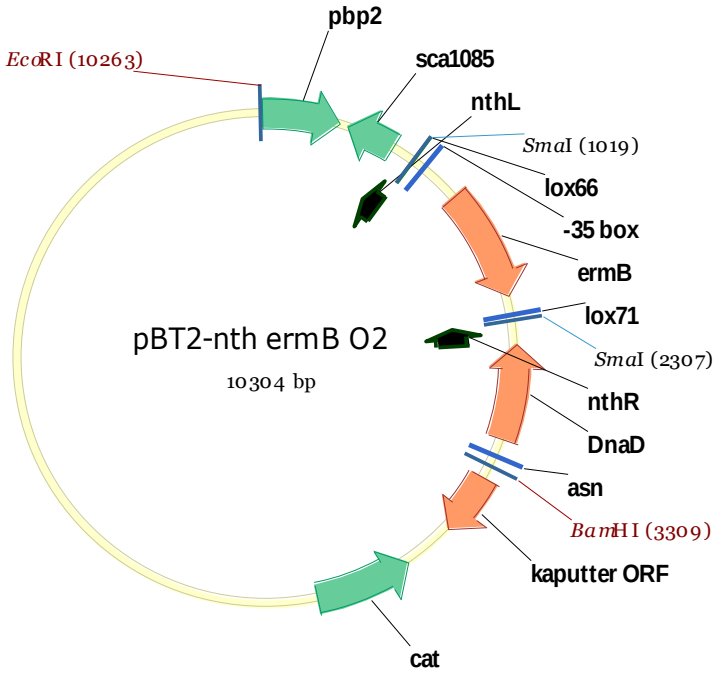


Figure 2. Construct of knock-out vector pBT2Δ*nthermB*

The erythromycin resistance cassette *erm*B replaced with *nth* partially and flanked parts (*nth*L/*nth*R) were left over. The plasmid encodes for a beta-lactamase (*bla*) and a chloramphenicol transferase (*cat*), and a temperature sensitive (ts) replication factor (*repF*).

Allelic replacement of the wild type *nth* gene was carried out with *erm*B in the reverse orientation (Fig. 3.) and the candidates which showing Em resistance and Cm sensitivity were selected.

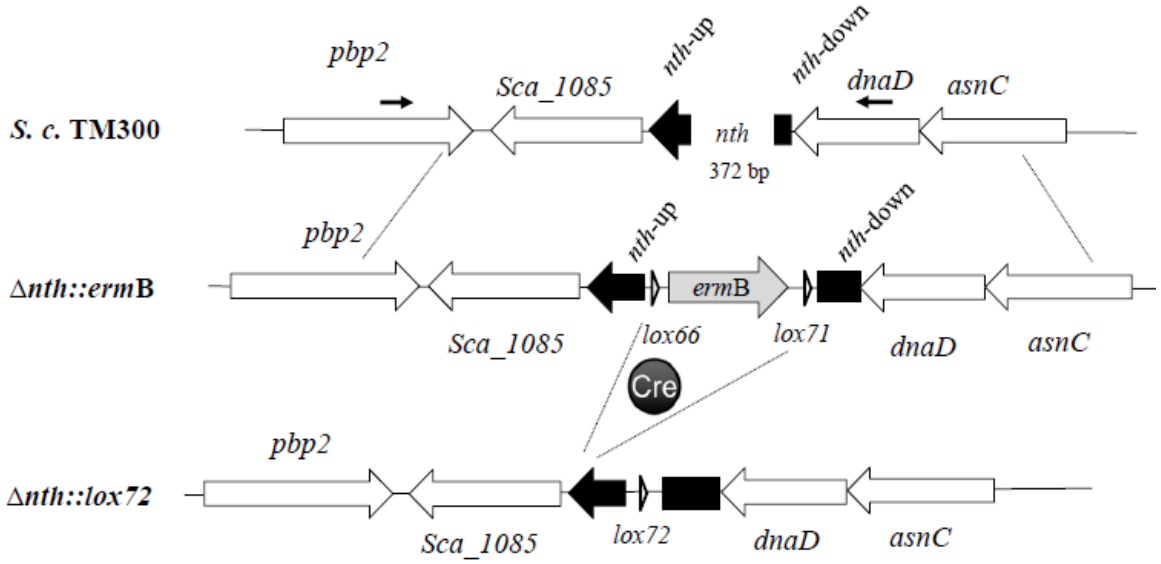


Figure 3. Allelic replacement of *nth* in *Staphylococcus carnosus* TM300

The resistance cassette of *erm*B in the vector is flanked by 1 kb regions found upstream and downstream of the *nth* gene in the genome. pBT2Δ*nth_erm*B was introduced into *S. carnosus* TM300 by protoplast transformation. The middle part of the *nth* gene (372 nt) from *S. carnosus* TM300 genomic DNA was replaced with *erm*B between pBT2Δ*nth_erm*B construct using homologous recombination method. Then, clones were screened for allelic replacement (chloramphenicol sensitivity and erythromycine resistance).

The colonies which carrying only *erm*B cassette with *lox66*/*71* sites on the chromosomal DNA were confirmed by PCR and sequencing. After transformation of pRAB1 vector to Δ*nth*::*erm*B protoplasts the resistance marker was removed by *Cre*-recombinase which recognized the corresponding *lox66*/*71* sites leaving the *lox72* site in the chromosome (Leibig *et al.*, 2008). pRAB1 was removed with temperature up-shift steps from the cells. Some Δ*nth* mutant candidates without any antibiotic resistance were selected and verified by sequencing (Fig. 4.).

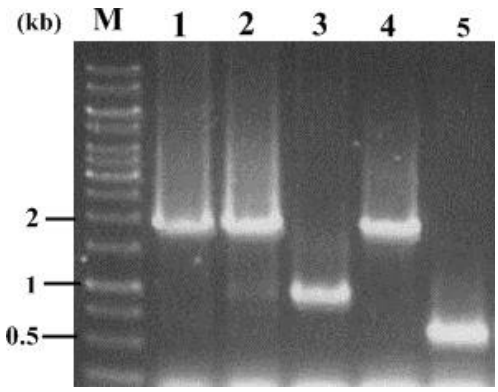


Figure 4. Verification of deletion mutants Δ*nth*::*erm*B and Δ*nth* lox72

EtBr stained 1% agarose gel demonstrating the gene deletion of *nth* and the subsequent deletion of *erm*B were verified in a PCR using primer pairs S1 and S2 with plasmids pBT2Δ*nth_erm*B/*E. coli* (1), pBT2Δ*nth_ermB*/*S. carnosus* TM300 (2) and genomic DNA of wild type *S. carnosus* TM300 (3), Δ*nth*::*erm*B (4) and Δ*nth lox*72 (5). M= 1 kb DNA ladder.

To create a complemented mutant strain, a xylose inducible vextor pTX30 (Strauss *et al.*, 1998) with tetracyline resistance was used. The lipase gene digested out from the pTX30 and *nth* was cloned instead of lipase gene (Fig. 5.). The constructed complementation vector pTX30*nth* was first transformed into electrocompetent *S. aureus* RN4220 cells by electroporation (Augustin & Götz, 1990). After isolation of pTX30*nth* from *S. aureus* RN4220 cells, it was transformed again to electrocompetent Δ*nth* mutant cells using electroporation (Löfblom *et al.*, 2007). Transformed vector was isolated again and sequence was verified by control digestions and sequencing. Hereby the complemented mutant Δ*nth*/pTX30*nth* was created.

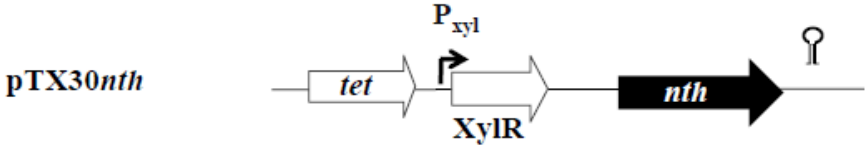


Figure 5. Linear map of *nth* expression vector pTX30*nth*

nth is transcribed from the xylose-inducible promoter using 0.5% xylose and Tetracyclin (25 µg/ml) in pTX30*nth*.

D.2 Observation of degradation capability

The total endonuclease activity of crude extracts from WT, Δ*nth* mutant and Δ*nth*/pTX30*nth* strains were investigated and compared using λ-DNA from *E. coli* as a

substrate. To eliminate the effect of exonucleases, cells were washed twice with medium and sonicated. The supernatant of cultures were used after centrifugation for the endonuclease assay.

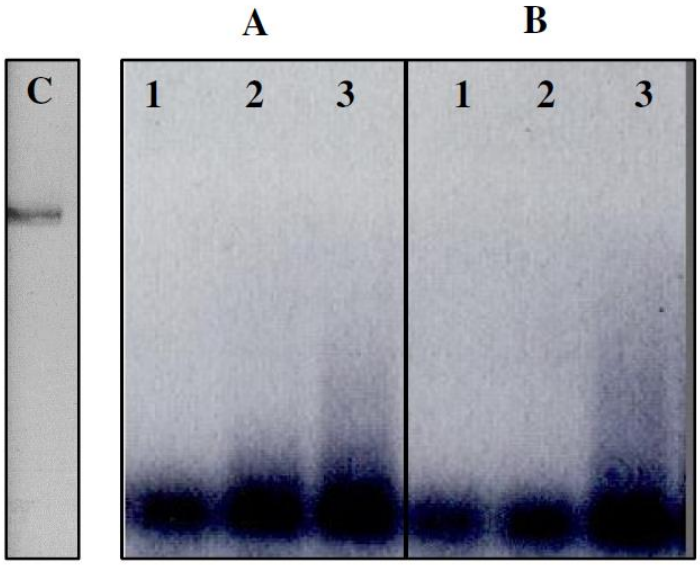


Figure 6. Degradation of undigested λ-DNA by crude extracts

EtBr stained 1% agarose gel showing the presence and absence of putative endonuclease III enzyme in total crude extracts. Therefore, 0.06 µg undigested λ-DNA from *E. coli* (C: Control) was mixed with crude extracts of wild type (1), Δ*nth*/pTX30*nth* (2) and Δ*nth* (3). The reaction times 60 min. (A) and 30 min (B). These results indicated that *nth* deletion in *S. carnosus* TM300 cause a significant decrease of total endonuclease activity.

The degradation activity of crude extracts showed that Δ*nth* mutant has an impaired digestion activity. However, the wild type and complemented mutant Δ*nth*/pTX30*nth* showed similar degradation capabilities (Fig. 6.).

D.3 Deletion of *nth* increases the sensitivity against oxidative stress

To verify the role of *nth* gene in oxidative damage response Δ*nth* mutant, wild type and Δ*nth*/pTX30*nth* cells were treated to H_2O_2 with different concentrations. In comparative experiments Δ*nth* mutant grew marginally slower at 37 °C compared to wild type and Δ*nth*/pTX30*nth* strains. Treatment with H_2O_2 (22-120 mM) revealed that Δ*nth* mutant was markedly more sensitive than the wild type and complemented mutant strains. As significant killing was observed at >45 mM H_2O_2 (Fig. 7.). In comparison, the wild type exhibited good tolerance to H_2O_2 and significant killing was only apparent at

concentrations >120 mM. These results confirms that *nth* is an important contributor to the oxidative damage DNA repair *in vivo*.

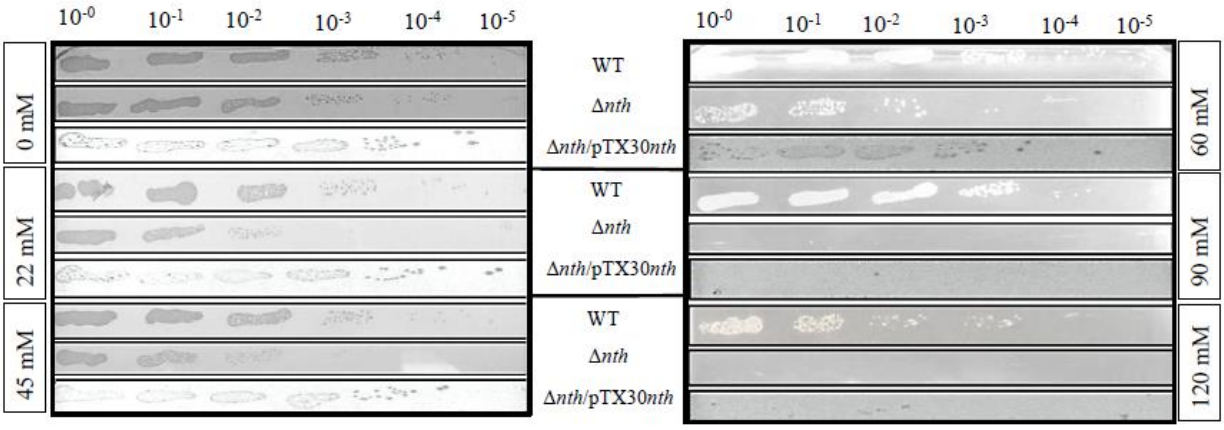


Figure 7. Deletion of *nth* increases sensitivity to H_2O_2

Samples of 10 µl from serial dilutions (from left to right: undiluted, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$) of WT, Δ*nth* mutant and Δ*nth*/pTX30*nth* control (0mM) and H_2O_2 exposed (22, 45, 60, 90 and 120 mM) cultures were spotted on BM plates but only Δ*nth*/pTX30*nth* cultures were spotted without glucose on BM plates with 0.5% xylose and 25 µg/ml tetracyclin.

D.4 Spontaneous mutation frequency of Δ*nth* mutant

We observed the mutation frequencies of the colonies grown on TSB-agar and TSB-agar without glucose plates in the presence of mutagenic streptomycine and mitomycin-C antibiotics. Using the microdilution method, the MICs of mitomycin-C and streptomycin were determined. The MIC of streptomycine was 1,55 µg/ml in the case of the wild type and the Δ*nth*/pTX30*nth*, whereas the MIC of Δ*nth* mutant was at 1,7 µg/ml. Beside, the MICs of mitomycin-C of all strains were 1,4 µg/ml (Tab. D.1).

Tab. D.1 Determined minimum inhibitory concentrations (MIC)

	MIC (µg/ml)	
Strain	Mitomycin-C	Streptomycin
S. c. TM300	1,4	1,55
S. c. Δ*nth*	1,4	1,7
S. c. Δ*nth*/pTX30*nth*	1,4	1,55

We observed that the *nth* deficient cells were more sensitive against mutagenic antibiotics like streptomycin and mitomycin-C compared to WT and complemented mutant cells. The number of survivor cells of WT and Δ*nth*/pTX30*nth* were 2-3 fold higher than Δ*nth* mutant cells after streptomycin or mitomycin-C treatment (Fig. 8.). These results showed that the mutagenic lesions can be repaired by endonuclease III.

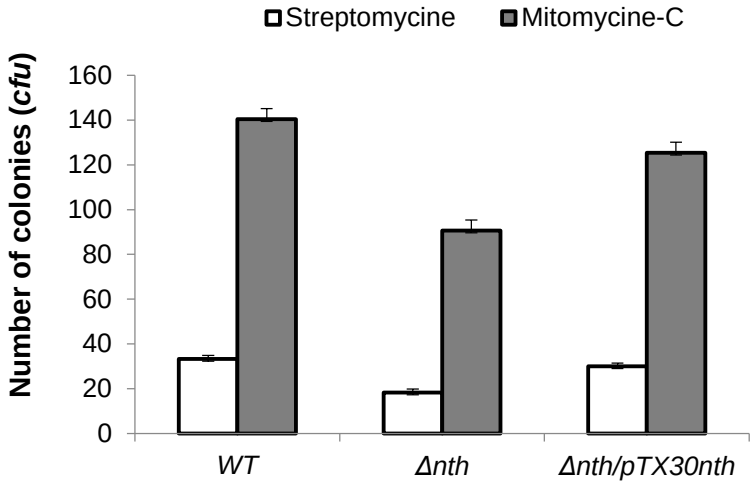


Figure 8. Resistance of spontaneous mutation against streptomycine and mitomycin-C

No significant difference was observed between the number of cells after treatment of streptomycine. The number of resistant WT and complemented mutant (Δ*nth*/pTX30*nth*) cells were almost equal against mitomycin-C, but the number of Δ*nth* mutant cells was approximately half as high.

D.5 Absence of *nth* improves the transformation efficiency

Comparison of transformation efficiencies were performed between wild type and Δ*nth* mutant cells. Electroporation was used as a transformation method and repeated three times. 2 µg of pBT2 shuttle vector isolated from *E. coli* DH5α was transformed to electrocompetent *S. carnosus* TM300 and Δ*nth* mutant cells (Löfblom *et al.*, 2007). Transformation was verified by isolation of pBT2 from positive candidates. The colonies of WT and Δ*nth* mutant were counted and compared for the transformation improvement. While the WT strain showed 5,8 x 10^2 *cfu*/ml plasmid uptake, the Δ*nth* mutant colonies were showed 18,5 x 10^2 *cfu*/ml (Fig. 9.).

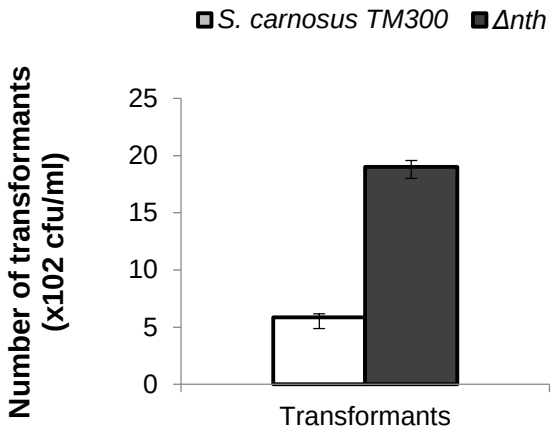


Figure 9. Comparison of transformation efficiencies

These results showed that the absence of *nth* increase the uptake foreign DNA into Δ*nth* mutant cells.

E. DISCUSSION

E.1 Inactivation and complementation of Δ*nth* mutant

The product of the gene *nth*, annotated as a putative endonuclease III, was inactivated by allelic replacement (Brückner, 1997) in *S. carnosus* TM300. Inactivated *nth* has important functions in processing genetic information, such as base excision repair and degradation of foreign nucleic acids during transformation process. The essential primosomal gene *dna*D, encoding for a protein with DNA-replication activity, is in the same operon with *nth*. In addition, *dna*D may play an important role in DNA repair in addition to its essential role in replication initiation (Collier *et al.*, 2012). Deletion of *nth* resulted in some phenotypical differences between the Δ*nth* mutant and WT strains. The lack of repair mechanisms which affect the number of living cells was quantitatively noticed and a clear effect of endonuclease III on foreign nucleic acids has been observed. In addition, a complemented mutant was generated to reconstitute the endonuclease activity. Therefore, the *nth* gene was ligated into a xylose inducible expression vector (pTX30) and transformed into the Δ*nth* mutant. After induction of *nth* with xylose, the repair and degradation activities of endonuclease III could be restored. Furthermore, the results verified that the absence of endonuclease III plays a critical role on cell viability.

E.2 Repair/degradation function of endonuclease III and their effects on transformation efficiency

DNA is often exposed to endogenous and exogenous agents which are able to modify bases, resulting in mutagenesis in the absence of DNA repair. Cellular DNA is frequently damaged by mutagens, oxygen radicals, ionising radiation, and even normal cellular metabolism creating mutagenic and cytotoxic DNA lesions. Cells depend on the corporated action of DNA repair enzymes which recognize and excise these DNA lesions. Most DNA damage in cells occurs to DNA bases, and the primary means of restoring the correct DNA base sequence is through the DNA base excision repair pathway (Lindahl & Wood, 1999). DNA damage occur unceasingly in all cells. Spontaneous DNA base loss, as well as the removal of damaged DNA bases by specific enzymes targeted to distinct base lesions, creates non-coding and lethal apurinic/apyrimidinic (AP) sites (Mol *et al.*, 2000). Endonuclease III plays a crucial role in the repair of DNA damage (Doetsch & Cunningham, 1990) with an apurinic (AP) lyase activity at apurinic/apyrimidinic sites in

DNA and it has a DNA glycosylase activity which excises purin and pyrimidine residues (Asahara *et al.*, 1989). In this study, we elucidated the protective role of endonuclease III in *S. carnosus* TM300 against mutagenic agents. Oxidative damage experiments using H_2O_2 indicated that Δ*nth* mutant was pretty much sensitive compared to WT strain. We know in literatures that base excision repair allows repair of DNA damages which are produced by oxidation, alkylation, deamination or hydroxylation of DNA bases (Krokan & Slupphaug, 1998). Oxidative damage to cellular components is an indispensable result of aerobic respiration. Reactive oxygen species produced as by-products of respiration cause mutations in the genome through oxidation of DNA bases (Lindahl & Wood, 1999). Collier et *al.* (2012) has been demonstrated that the deletion of the *nth* in *Bacillus subtilis* strain 168 resulted an increased sensitivity to H_2O_2 exposure, suggesting that *nth* plays a prominent role in the oxidative damage response (Collier *et al.*, 2012). Accordingly, we observed that *nth* deficient *S. carnosus* TM300 cells were more sensitive against H_2O_2. After treatment with H_2O_2, the number of Δ*nth* mutants was reduced compared to the WT and complemented mutant strains. According to the regulation of transcription of *nth*, Collier *et al.* were suggested that the regulation mechanism of *nth* transcription could be accommodate a direct adjustment of the activity of definitive DNA repair enzymes such as *nth* related in BER. The genetic link and functional cooperation of *dna*D with *nth* (both genes are juxtaposed in the same operon) offers an additional H_2O_2-mediated regulatory level and only modulating oxidative damage induced BER (Collier *et al.*, 2012).

DNA uptake has been characterized to various extents in both Gram-positive, particularly *Bacillus subtilis* and *S. pneumoniae*, and Gram-negative bacteria, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Dubnau, 1999). The effects of nuclease on the efficiencies of transformation, including electroporation and conjugation have been examined in an *E. coli* recombinant strain expressing *V. vulnificus* nuclease (Vvn). The role of Vvn in preventing uptake of foreign DNA in *V. vulnificus* was also determined by isolating a Vvn-deficient *V. vulnificus* mutant and comparing its frequencies of transformation and conjugation with those of the parent strain (Wu *et al.*, 2001). It is well known that most bacteria carry a specific restriction modification (R–M) system which acts as a barrier against the invasion of exogenous DNA (Arber & Linn, 1969). It is of interest that *S. carnosus* TM300 does not possess a functional R-M system because the necessary genes are missing (Seeber *et al.*, 1990). Nevertheless, transformation of foreign nucleic acids into *S. carnosus* TM300 cells is very efficient. We know that the

structure of bacterial cell wall and secreted exogenous/endogenous nucleases can decrease the transformation efficiency. We demonstrated a clear increase of transformation efficiency when the putative endonuclease III was deleted from *S. carnosus* TM300 genome. With this deletion we could scale up the number of transformants about three fold compared to WT strain.

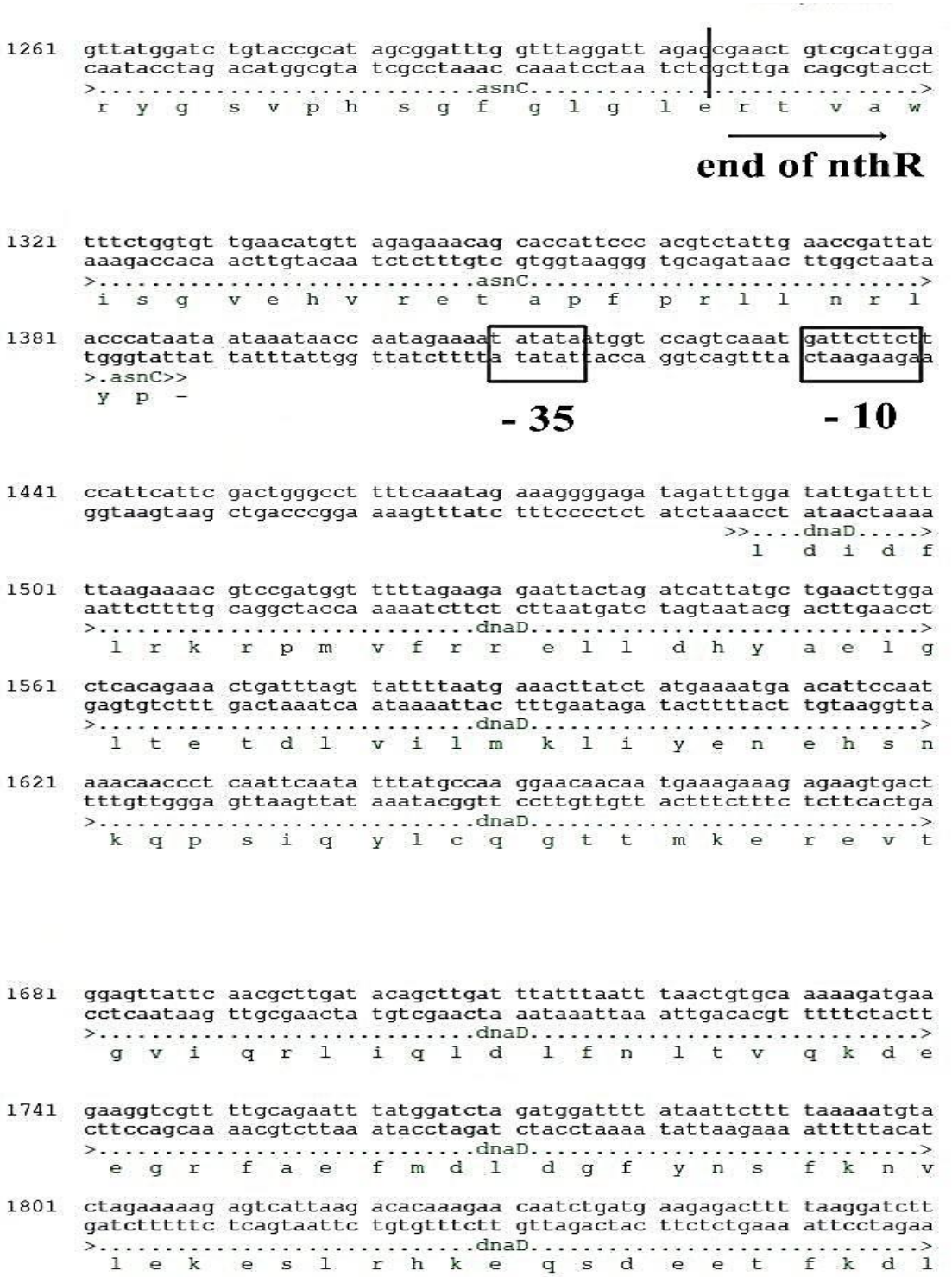
Nuclease activity were tested by mixing of crude extracts from Δ*nth* mutant, complemented mutant and WT strains with undigested λ-DNA from *E. coli*. The results indicates clear degradation differences between strains. The degradation capability of foreign DNA in Δ*nth* mutant cells were quite lower in compare to complemented mutant and WT strain.

E.3 Resistance against spontaneous mutagenesis by using mutagen antibiotics

Mitomycin-C is a DNA synthesis inhibitor. It reacts covalently with DNA, *in vivo* and *in vitro*, forming cross-links between the complementary strands of DNA. This interaction prevents the separation of the complementary DNA strands, thus inhibiting DNA replication. Mitomycin-C is one of the few antibiotics known to interact with DNA in this fashion (Ueda *et al.*, 1983). On the other hand, streptomycin is an antibiotic which inhibits initiation, elongation and termination of protein synthesis in prokaryotes. It binds to the 23S rRNA of the 30S ribosomal subunit. This protein appears to be responsible (in the absence of streptomycin) for the binding of mRNA (Sharma *et al.*, 2007). Both mutagenic antibiotics have been used for testing of spontaneous mutagenic responses of Δ*nth* mutant cells. Therefore cells were treated with determined MIC values for the mutagenesis analysis. Cunningham and Weiss were reported that a Δ*nth* mutant is known to have a weak mutator effect in *E. coli* cells (Cunningham & Weiss, 1985). Additionally, Saito *et al.* were measured the mutations in rifampin-sensitive to rifampin-resistant and nalidixic acid-sensitive to nalidixic acid-resistant *E. coli* cells (Saito *et al.*, 1997). Their results further suggest that the spontaneous mutagenic lesions can be repaired mainly by endonuclease III. Also, we have been demonstrated that *nth* deficient cells were more sensitive against some mutagenic antibiotics like streptomycin and mitomycin-C compared to WT and complemented mutant strains. The survived number of WT and complemented mutant cells were 2-3 fold higher than the Δ*nth* mutant cells after streptomycin or mitomycin-C treatment. These results indicates that the mutagenic lesions can be repaired by endonuclease III.

F. SEQUENCES

F.1 Gene sequence of *sca_1086* encoding for *nth* of *S. carnosus* TM300



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        f  q  f    i  e  q    s  f  g  r    p  l  s    p  i  e    i  d  t  l

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      ttagttacct atctacaact attagtactg tatagacaat atgttcgacg tcacttactt
      >.............................dnaD.............................>
        n  q  w    i  d  v    d  n  h  d    i  s  v    i  q  a    a  v  n  e

1981  gcattaagtc aagagaaaat taatttcaaa tacatcgatc gaatattatt gaattggaaa
      cgtaattcag ttctctttta attaaagttt atgtagctag cttataataa cttaaccttt
      >.............................dnaD.............................>
        a  l  s    q  e  k    i  n  f  k    y  i  d    r  i  l    l  n  w  k

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      ttcttattac agttttgtca gctactaagg ttttttttagt cggttgttat agtatgcggt
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                                                                SD
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      gtttacttcg tatgacattt tttatatgga tttaagctga ccaatttact cctcctgagt
      >.............................dnaD.............................>
        q  m  k    h  t  v    k  n  i  p    k  f  d    w  l  n    e  e  d  s

2161  taaatgttaa gcaaaaagaa agcattaagt atgattgatg taattgctga tatgtttcca
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      >>> dnaD
        -
         >>.............................nth.............................>
           m  l    s  k  k    k  a  l  s    m  i  d    v  i  a    d  m  f  p

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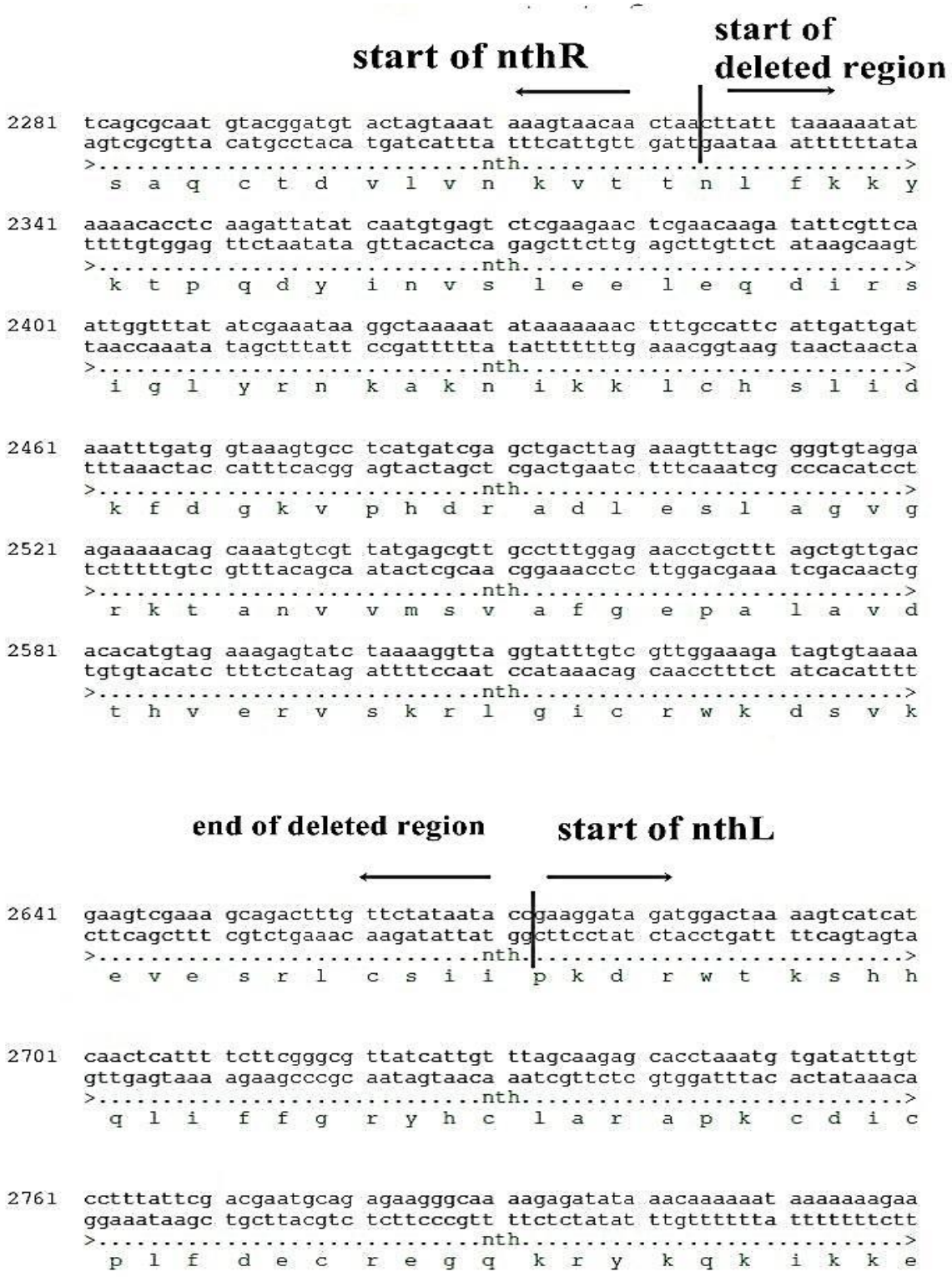
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start of deleted region
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>.....nth.....>
s a q c t d v l v n k v t t n l f k k y
2341 aaaacacctc aagattatat caatgtgagt ctcgaagaac tcgaacaaga tattcgttca
ttttgtggag ttctaatata gttacactca gagcttcttg agcttgttct ataagcaagt
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2401 attggtttat atcgaaataa ggctaaaaat ataaaaaaac tttgccattc attgattgat
taaccaaata tagctttatt ccgattttta tatttttttg aaacggtaag taactaacta
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i g l y r n k a k n i k k l c h s l i d
2461 aaatttgatg gtaaagtgcc tcatgatcga gctgacttag aaagtttagc gggtgtagga
tttaaactac catttcacgg agtactagct cgactgaatc tttcaaatcg cccacatcct
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>.....nth.....>
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tgtgtacatc tttctcatag attttccaat ccataaacag caacctttct atcacatttt
>.....nth.....>
t h v e r v s k r l g i c r w k d s v k
end of deleted region
start of nthL
2641 gaagtcgaaa gcagactttg ttctataata ccgaaggata gatggactaa aagtcatcat
cttcagcttt cgtctgaaac aagatattat ggcttcctat ctacctgatt ttcagtagta
>.....nth.....>
e v e s r l c s i i p k d r w t k s h h
2701 caactcattt tcttcgggcg ttatcattgt ttagcaagag cacctaaatg tgatatttgt
gttgagtaaa agaagcccgc aatagtaaca aatcgttctc gtggatttac actataaaca
>.....nth.....>
q l i f f g r y h c l a r a p k c d i c
2761 cctttattcg acgaatgcag agaagggcaa aagagatata aacaaaaaat aaaaaaagaa
ggaaataagc tgcttacgtc tcttcccgtt ttctctatat ttgttttta ttttttctt
>.....nth.....>
p l f d e c r e g q k r y k q k i k k e

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      >.....nth.....>>
        a  e  k    s  -
                                    >>............Sca_1085.............>
                                      m   i  a  k   s  d  f   e  k  r  e
```

SD (sca_1085)

```
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2941  cttgatgcat attttaattt actgcaaagt tatttttgcg aaatcaataa tatatctaat
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        l  d  a   y  f  n   l  l  q  s   y  f  c   e  i  n   n  i  s  n

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3061  gaatatatta atgagcgttg tcatcatttc atgggatatc gacagatgaa aaccttagct
      cttatataat tactcgcaac agtagtaaag taccctatag ctgtctactt ttggaatcga
      >............................Sca_1085.............................>
        e  y  i   n  e  r   c  h  h  f   m  g  y   r  q  m   k  t  l  a

3121  tctgaactaa ttaaaatgaa tgctgcttat aaaacgcgac aagctcataa aaaacaataa
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                                                  <<....pbp2.....<
                                                    -   s  a  k  d

3241  attgaaaatt gcattaaggt taaagaattt agttaatgca tttccgcttt gttgattatt
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        n  f  i   a  n  l   n  f  f  k   t  l  a   n  g  s   q  q  n  n

3301  attgccttgg ctattattac cttgttgatt attattgcct tggctgttat taccttgttg
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      <.............................pbp2.............................<
        n  g  q   s  n  n   g  q  q  n   n  n  g   q  s  n   n  g  q  q

3361  attgctttgt ccagaactat tatttgaacc actgttacta ctgctgctgc caccatttac
      taacgaaaca ggtcttgata ataaacttgg tgacaatgat gacgacgacg gtggtaaatg
      <.............................pbp2.............................<
        n  s  q   g  s  s   n  n  s  g   s  n  s   s  s  s   g  g  n  v
```

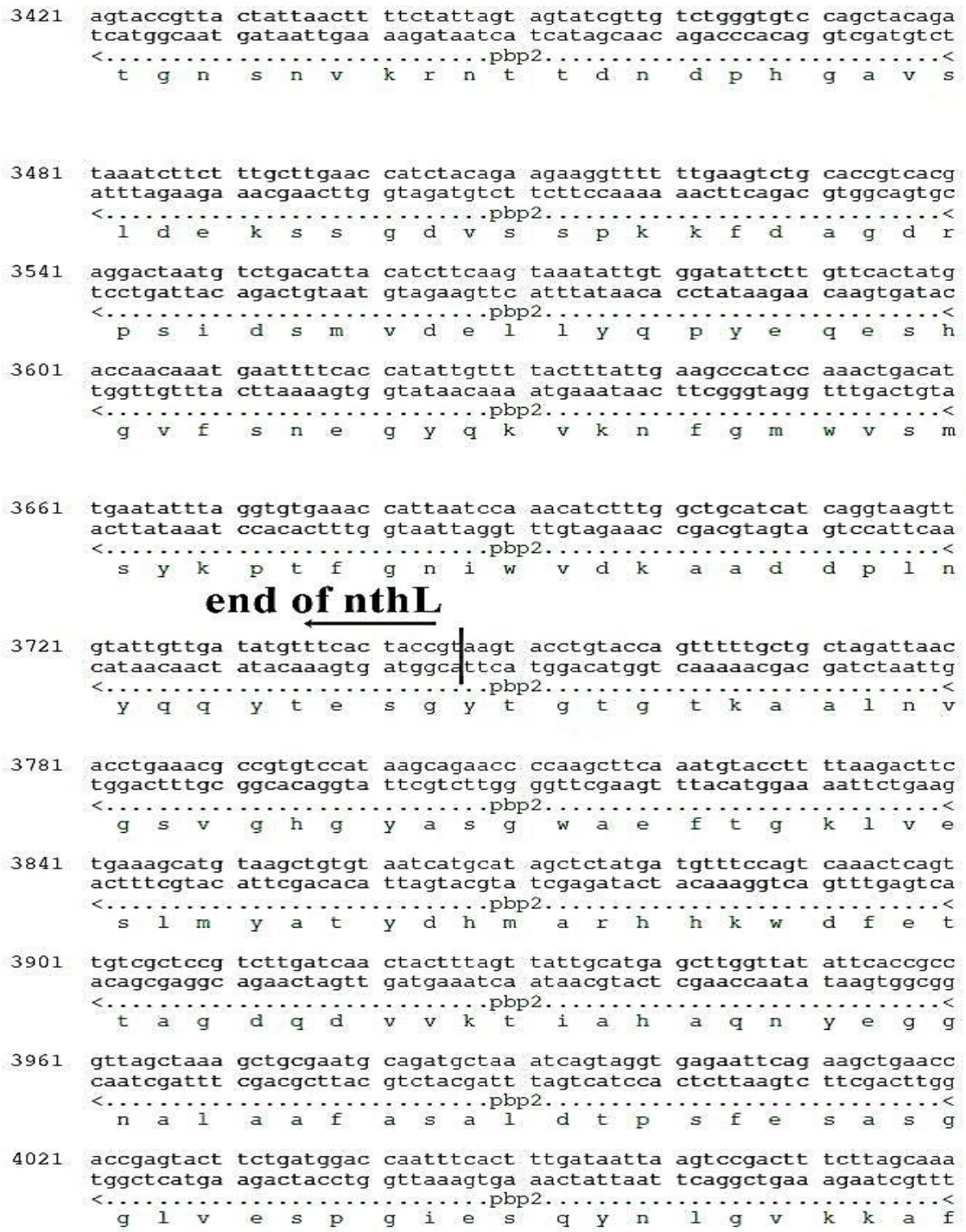


Figure 10. Nucleotide sequence of *sca_1086* encoding for *nth* from *S. carnosus* TM300

The deviated amino acid sequences are illustrated below and above the nucleotide sequences. The putative Shine Dalgarno sequence (SD) shown in rectangle. The partial gene deletion is highlighted, such as *nthL* and *nthR* depict the actual undeleted parts of *nth*. Accession number YP_002633494, GI:224475888 NCBI GenBank.

CHAPTER II

Investigation and characterization of a putative *PII-like* protein in *Staphylococcus aureus*

A. Abstracts

A.1 English abstract

PII proteins are signal transduction proteins in bacteria, archaea and plants, which coordinate and regulate many aspects of nitrogen metabolism by interacting with enzymes, transcription factors and membrane transport proteins. The genome of *Staphylococcus aureus* NCTC8325 does not encode for a homologue of a canonical PII protein. However, the gene SAOUHSC_00452, annotated as a hypothetical protein, encodes a protein, which has predicted structural similarity to canonical PII and is therefore termed as *PII-like* protein.

In this study, we generated a *PII-like* deletion mutant (Δ*PII-like*) in *S. aureus* NCTC8325 and studied its function by comparative phenotypic analysis. Deletion of the putative *PII-like* gene resulted in severe impairment during the exponential growth phase in nitrogen deficient and excess media. In addition, the Δ*PII-like* mutant exhibited an increased biofilm formation compared to the wild type strain. These results indicated a possible role of *PII-like* in central metabolic pathways, which had to be elucidated.

We investigated the thermodynamic binding properties of *PII-like* protein to various molecules such as ATP, ADP, AMP, GTP, TTP, cAMP and cyclic diadenosine monophosphate (c-di-AMP) using Isothermal Titration Calorimetry (ITC) and Biacore Surface Plasmon Resonance (SPR). The bacterial second messenger c-di-AMP was shown to bind tightly to *PII-like*, whereas cAMP, another second messenger molecule, just interacted loosely with *PII-like*. Unfortunately, we could not observed any binding interactions between ATP, ADP, AMP, GTP and TTP.

A.2 Deutsche Zusammenfassung (German abstract)

PII-Proteine stellen wichtige Signaltransduktionproteine in Bakterien, Archaeen und Pflanzen, dar, welche viele Aspekte des Stickstoff-Stoffwechsels koordinieren und regulieren, indem sie mit Enzymen, Transkriptionsfaktoren und Membrantransportproteine interagieren. Im Genom von *Staphylococcus aureus* NCTC8325 befindet sich kein zu einem kanonischen PII-Protein homologen Gen. Das als –hypothetisches Protein SAOUHSC_00452 Gen, annotierte Gen, kodiert für ein Protein, das strukturelle Ähnlichkeit mit PII aufzeigt und wird daher als PII-ähnliches (*PII-like*) Protein bezeichnet.

In dieser Studie wurde eine *PII-like*-Deletionsmutante (Δ*PII-like*) in *S. aureus* NCTC8325 hergestellt und seine Funktion durch vergleichende phänotypische Analysen studiert. Die Deletion des vermeintlichen *PII-like*-Gens führte zu einer schweren Beeinträchtigung während der exponentiellen Wachstumsphase in einem Stickstoff Mangelmedium und Überschussmedium. Außerdem zeigte Δ*PII-like* eine gesteigerte Biofilmbildung im Vergleich zum Wildtyp-Stamm. Dieses Ergebnis weist auf eine mögliche Rolle von *PII-like* in zentralen Stoffwechselprozessen hin, die aufgeklärt werden sollten.

Wir untersuchten die thermodynamischen Bindungseigenschaften von *PII-like*, mit verschiedenen Molekülen wie ATP, ADP, AMP, GTP, TTP, cAMP und zyklischem Diadenosin-Monophosphat (c-di-AMP) mit der isothermen Titrationskalorimetrie (ITC) und der Biacore Oberflächenplasmonenresonanz (SPR). Der bakterielle Second messenger c-di-AMP zeigte ein signifikantes Bindungssignal, wohingegen ein weiteres zweites Second messenger- Molekül, das cAMP nur eine schwache Interaktion mit *PII-like* einging. Leider konnten wir keine Wechselwirkungen zwischen den Nukleotiden ATP, ADP, AMP, GTP und TTP mit *PII-like* beobachten.

B. INTRODUCTION

B.1 Importance of Nitrogen

Nitrogen is one of the most important nutrients for all known forms of life and its crucial in maintaining an optimal growth rate. It is required to build a variety of vital building blocks such as amino acids, nucleotides or amino sugars. The abundance of nitrogen sources ranging from ammonium (NH_4^+) up to atmospheric nitrogen (N_2) (Reitzer, 2003). Ammonia (NH_4^+) is the preferred form of nitrogen and assimilated into two amino acids, glutamate and glutamine. These two amino acids are used as a source of nitrogen for other nitrogen-containing biomolecules. Therefore, one or both of these amino acids are present in most cells, at concentrations higher than other amino acids. In addition, in order to maintain an osmotic balance between the cytosol and the external medium, the regulation of the intracellular nitrogen concentration is important (Reitzer & Magasanik, 1985).

B.2 PII system

A central regulatory system of nitrogen assimilation is based on the family of PII proteins. This protein family consists of three subfamilies, named GlnB, GlnK and NifI, acting through recording and routing of signals that affect the cellular nitrogen status (Arcondeguy *et al.*, 2001). They are among the most highly conserved and most widely used signal transduction proteins found in bacteria, archaea and in the plastids of plants (Huergo *et al.*, 2013).

B.2.1 The biochemistry of PII protein

The PII protein was first discovered in 1969 during the analysis of the regulation of glutamine synthetase in *E. coli* (Shapiro, 1969) and was designated as GlnB. In 1995, a second PII paralog was discovered in *E. coli*, which was designated as GlnK (van Heeswijk *et al.*, 1995, van Heeswijk *et al.*, 1996, Forchhammer, 2008).
The proteins of the PII family are approximately 110 to 120 amino acids in size and highly conserved across its entire sequence. The average molecular mass of PII proteins is approximately 12.5 kDa. Crystallization studies, sedimentation equilibrium and gel

filtration experiments have been shown that PII proteins exist as a homotrimeric structure (Fig. 11.) (Forchhammer & Tandeau de Marsac, 1994, Leigh & Dodsworth, 2007).

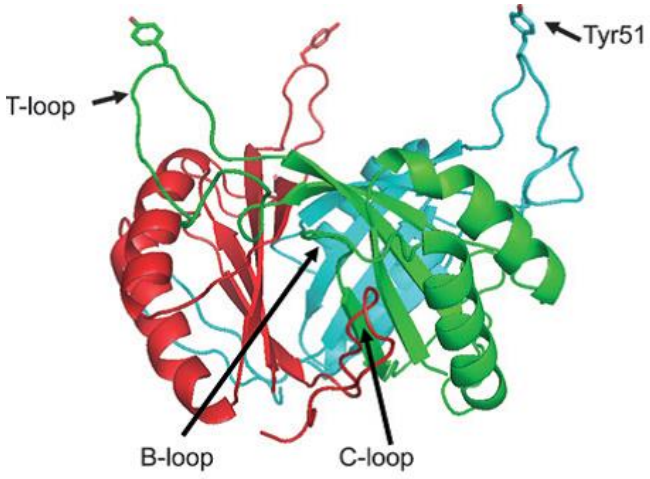


Figure 11. PII protein structure from *Escherichia coli*

The arrows indicate the B, C and T loops in the absence of any ligand and the side chain of Try51 which has been described as the site of reversible uridylylation in many proteobacteria PII (Huergo et al., 2013).

Each monomer of a PII trimer comprises of two α-helices and four β-strands arranged so that the α-helices and β-strands form a double βαβ motif connected by a large loop of 19 amino acids (Fig. 11.). This large loop contains a post-translational site, was named as *T-loop* and it was first investigated in *E. coli*, where residue *Tyr51* is subject to uridylylation (Son & Rhee, 1987). The *B- loop* is a smaller loop which is located between the second α-helix and the fourth β-strand, and the *C-loop* is located at the C-terminus (Huergo *et al.*, 2013).

B.2.2 Ligand binding and modification of PII proteins

The energy signal of ATP, the carbon signal of 2-oxoglutarate (2-OG) and the nitrogen signal of glutamine through glutamine-dependent uridylylation/deuridylylation are potential respond of central metabolic signals of PII proteins (Forchhammer, 2004). The signal perceived by PII occurs through the binding of small effector molecules, mainly ATP and α-ketoglutarate (Kamberov *et al.*, 1995, Forchhammer & Hedler, 1997). Co-crystals of *E. coli*'s GlnB and GlnK with ATP revealed three ATP-binding sites in the lateral furrows between the subunits (Xu *et al.*, 1998, Xu *et al.*, 2001). The effector molecule

ATP comes over the entire length of the furrow with GlnK into contact and binds to the residues of both subunits. The binding of ATP and α-ketoglutarate to the PII proteins depends strongly from one another (Kamberov *et al.*, 1995, Forchhammer & Hedler, 1997, Smith *et al.*, 2004). Moreover, it turned out that the ɣ-phosphate of ATP is essential for the interaction of effector molecules (Benelli *et al.*, 2001, Ruppert *et al.*, 2002). Although the α-ketoglutarate binding site has not been shown to date by crystal studies, a model could be developed through structural comparisons with other α-keto acid-binding proteins. This model suggests that α-ketoglutarate could bind in the cleft between B and C loop near the ɣ-phosphate of ATP (Benelli *et al.*, 2001).

In addition to the ability of PII proteins to bind ligands, PII proteins can be modified covalently in response to a change in the nitrogen availability. In all the cases studied, this modification occurs at an amino acid residue, which is located near the tip of the long and flexible T-loops (Xu *et al.*, 2003). To date, different types of modifications have been identified in various organisms. In proteobacteria, PII proteins are uridylylated at tyrosine residue 51 (Atkinson & Ninfa, 1999, Jiang *et al.*, 1998). Although the process of tyrosine modification is widely used, it is not universal. The PII protein in Cyanobacteria is phosphorylated in contrast to the enteric bacteria at the serine 49 under nitrogen deficiency or excess carbon (Forchhammer & Tandeau de Marsac, 1995). PII proteins, in some organisms do not seem to be modified, such as in Firmicutes (Wray *et al.*, 1994, Detsch & Stulke, 2003), in the cyanobacterium Prochlorococcus genus (Palinska *et al.*, 2002) or in plants (Moorhead & Smith, 2003, Smith *et al.*, 2004).

B.2.3 *PII-like* protein

Arconde´guy et *al.* (2001), proposed a PII nomenclature to create a rational framework for describing members of the PII family. They proposed three major groups, which called *gln*B, *gln*K, and *nif*I. These groups are based on two criteria: conservation of gene linkage and similarity of amino acid sequence level (Arcondeguy *et al.*, 2001). However, some other *PII-like* encoding genes did not fit the current classification. A few years later, Sant'Anna et *al.* (2009) have been evaluated the fitness of the previous classification bioinformatically and proposed a new probable member of this superfamily. They divided the PII superfamily again into three different groups based on operon structure and sequence features; GlnB-GlnK (GlnB/K), the NifI and an uncharacterized PII-New group (PII-NG) (Sant'Anna *et al.*, 2009). Multiple alignments using bioinformatical databases

showed, that the *PII-like* protein in *S. aureus* possessed over 30% similarity and 15% identity between a distant cyanobacterial strain of *Synechococcus elongatus* PCC 7942 (Synpcc7942_0321) (Fig. 12).

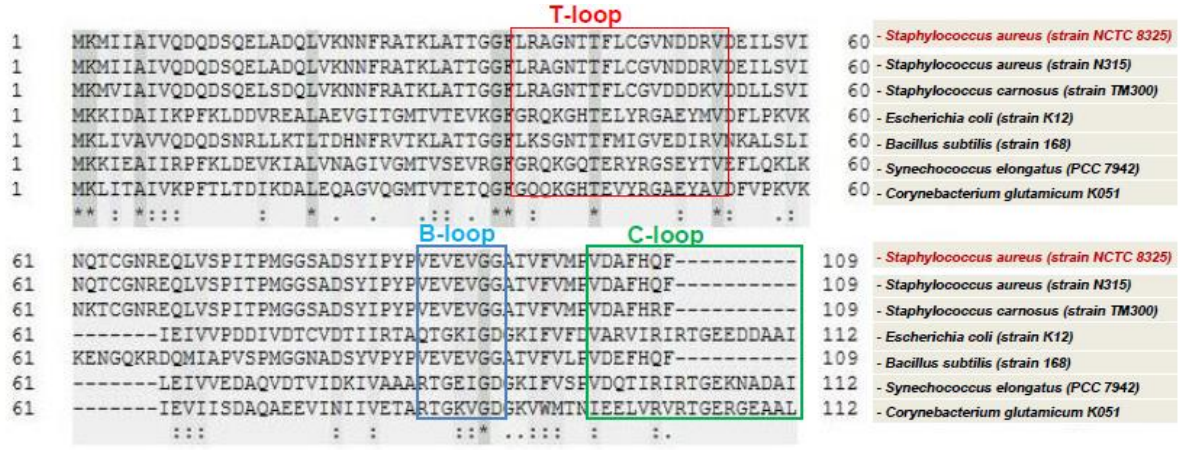


Figure 12. Multiple alignment of PII and putative *PII-like* proteins

Homology between *Staphylococcus aureus* (SAOUHSC_00452), *Bacillus subtilis* (BSU00290), *Synechococcus elongatus* PCC 7942 (Synpcc7942_0321), *Corynebacterium glutamicum* (NCgl1982) strains.

B.3 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive pathogen causing a majority of suppurative infections (Ghuysen & Strominger, 1963) and both community-acquired and nosocomial sepsis (Emori & Gaynes, 1993, Steinberg *et al.*, 1996). Studies have demonstrated that three nasal carriage patterns can be distinguished in the healthy adult population, ~20% of individuals are persistent *S. aureus* carriers; ~60% are intermittent carriers; and 20% are persistent non-carriers (Kluytmans, 1998). Furthermore, it is a model microorganism for structural and functional studies of the Gram-positive cell wall. *Staphylococcus aureus* is a major pathogen of increasing importance due to the rise in antibiotic resistance (Lowy, 1998).

B.4 Nitrogen regulation in *Staphylococcus aureus*

One of critical bacterial survival strategy is the nitrogen metabolism and is therefore tightly regulated in different steps. The first nitrogen donors in bacteria are glutamate and glutamine. Glutamine is crucial for osmotic balance in cells (Anderson & Witter, 1982). GlnR and TnrA are two repressors in *B. subtilis*, which are involved in glutamine metabolism (Schreier *et al.*, 1989, Wray *et al.*, 1996). Together GlnR and TnrA regulate

gene expression in response to the availability of nitrogen (Sonenshein, 2007). Despite its significance for nitrogen regulation of *B. subtilis*, a TnrA homologue could not be identified in staphylococci. Importantly, inactivation of *glnR* increased methicillin sensitivity by altering peptidoglycan synthesis and structure in methicillin resistance *S. aureus* (MRSA) (Gustafson *et al.*, 1994).

S. aureus can grow in a low-oxygen environment by fermentation or nitrate respiration (Burke & Lascelles, 1975). Therefore, adaptation to different levels of oxygen, nitrogen, or other electron acceptors is very important. The two-component **S**taphylococcal **r**espiratory **r**esponse AB (SrrAB) system is the major regulatory system responsible for anaerobic gene regulation (Pragman *et al.*, 2004). Another two-component system is the NreBC system which was first identified in *S. carnosus*, regulates nitrogen metabolism. NreB is a sensor kinase and NreC is a response regulator. The histidine kinase activity of NreB is increased when the cells are under low oxygen concentration and it activates the response regulator NreC. In addition, this system activates genes which are involved in nitrogen metabolism including *narT* (nitrite and nitrate transport), *nar*GHJI (respiratory nitrate reductase), and *nas*DE (nitrite reductase). The *nar*GHJI operon encodes the membrane-bound respiratory nitrate reductase which reduces nitrate to nitrite, and nitrite can be further reduced to ammonia by nitrite reductase (*nas*DE) (Schlag *et al.*, 2008, Schlag *et al.*, 2007).

B.5 PstA and DarA; *PII-like* as receptor proteins for cyclic diadenylate monophosphate (c-di-AMP)

Nowadays, there is no more knowledge about the properties and functions of *PII-like* proteins in pathogenic *S. aureus* strain. Corrigan et *al.* (2013) identified a *PII-like* protein as c-di-AMP binding protein in *S. aureus* subsp. aureus COL (MRSA) by using a genome-wide nucleotide protein interaction screen (DRaCALA) and renamed PstA (*PII-like* signal transduction protein A). PstA is able to bind diverse ligands such as ATP, ADP, c-di-AMP (Fig. 13.) and act by protein-protein interaction to control the activity of enzymes, transcription factor, or transport proteins. Additionally, PstA shares very low sequence similarity with classical PII proteins belonging to the GlnB/K group. Corrigan et *al.* (2013) assumed that upon c-di-AMP binding or release the *S. aureus* PstA protein interacts with other cellular proteins. However, this still needs to be elucidated (Corrigan *et al.*, 2013).

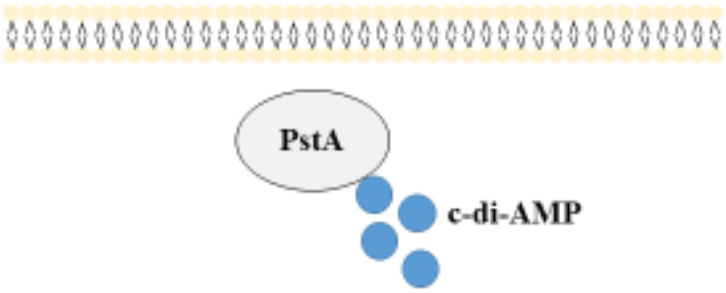


Figure 13. Binding of PstA protein with c-di-AMP in *S. aureus*

(adapted from (Corrigan & Grundling, 2013)

Up to date, crystal structures of several GlnB and GlnK proteins with/without bound ligand have been reported from different microorganisms. These structures showed that PII proteins have a highly conserved three-dimensional homo-trimeric structure (Conroy *et al.*, 2007, Nichols *et al.*, 2006, Sakai *et al.*, 2005, Zhao *et al.*, 2010, Xu *et al.*, 2001).
Müller et *al.* (2014) reported that PstA includes a ferredoxin-like fold and is structurally related to a class of PII signal-transduction proteins, that is mainly involved in nitrogen metabolism. They studied the molecular details of c-di-AMP binding biochemically *via* surface plasmon resonance experiments (BiacoreX100) and crystallized *S. aureus* PstA in the presence and absence of the cyclic dinucleotide (Fig. 14.-B) (Müller *et al.*, 2015). Gundlach et *al.* (2014) have been identified a *PII-like* protein in *Bacillus subtilis* as a c-di-AMP receptor protein, which was named DarA. They showed that DarA is capable to bind c-di-AMP and, with lower affinity, also 3'3'-cGAMP, but not c-di-GMP or 2'3'- cGAMP. Furthermore, crystal structure analysis revealed a high homology between DarA and PII signal transducer proteins. According to these results, they demonstrated that DarA has a homo-trimeric structure which binds three molecules of c-di-AMP and each in a pocket located between two subunits (Fig. 14.-A) (Gundlach *et al.*, 2014). In addition, Campeotto et *al.* (2015) crystallized the PstA/c-di-AMP complex structure and they reported three identical c-di-AMP binding sites per trimer with each binding site at a monomer-monomer interface. Structural analysis of PstA, together with bioinformatical data, showed that the protein belongs to a new family of proteins with a similar core fold but with distinct features to classical PII proteins (Fig. 14.-C) (Campeotto *et al.*, 2014).

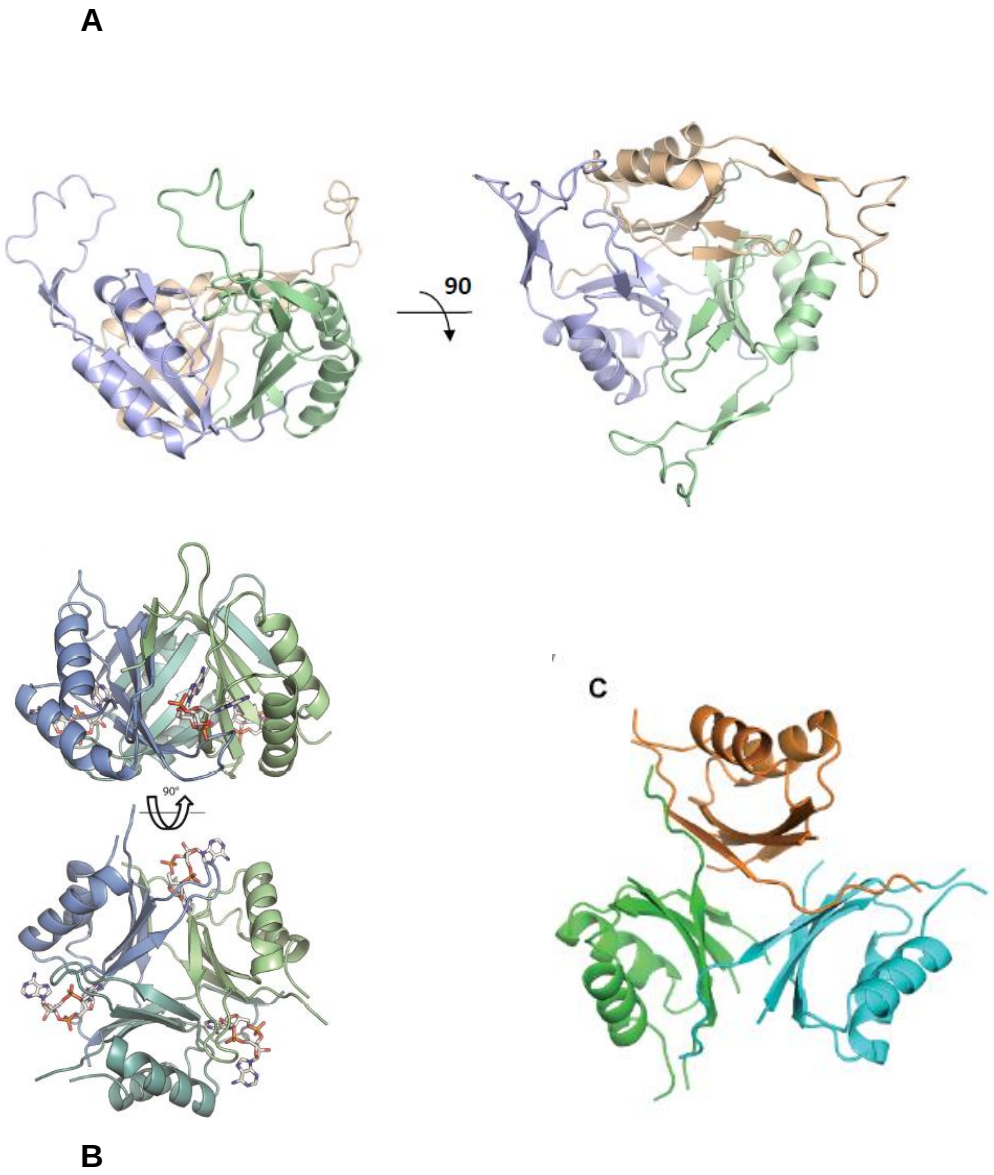


Figure 14. Crystal structure complexes of PstA and DarA

A) Two perpendicular views of DarA crystal structure complex with c-di-AMP from *B. subtilis*. The three subunits of homotrimeric DarA are colored blue, green and beige (Gundlach *et al.*, 2014), B) PstA trimer from *S. aureus* with bound c-di-AMP in side (upper part) and bottom views (lower part). The c-di-AMP moieties are deeply buried in the inter-subunit clefts (Müller *et al.*, 2015), C) Structure of PstA trimer from *S. aureus* with the individual monomers shown in different colors (Campeotto *et al.*, 2014).

B.6 Nucleotides as signaling molecules (seconder messengers)

Various nucleotide derivatives are important *second messengers*. In all organisms, cellular functions are regulated through these second messenger molecules. The linear

(p) ppGpp acts as alarm-on in the stress response to limitation of amino acids, phosphate, fatty acids, carbon and iron by different mechanisms (Persky *et al.*, 2009, Lapouge *et al.*, 2008, Vinella *et al.*, 2005). Furthermore, bacteria use a variety of cyclic nucleotide derivatives. These are the cyclic mononucleotides cAMP and cGMP as well as the cyclic dinucleotide c-di-GMP, c-di-AMP and cGAMP (Fig. 15.).

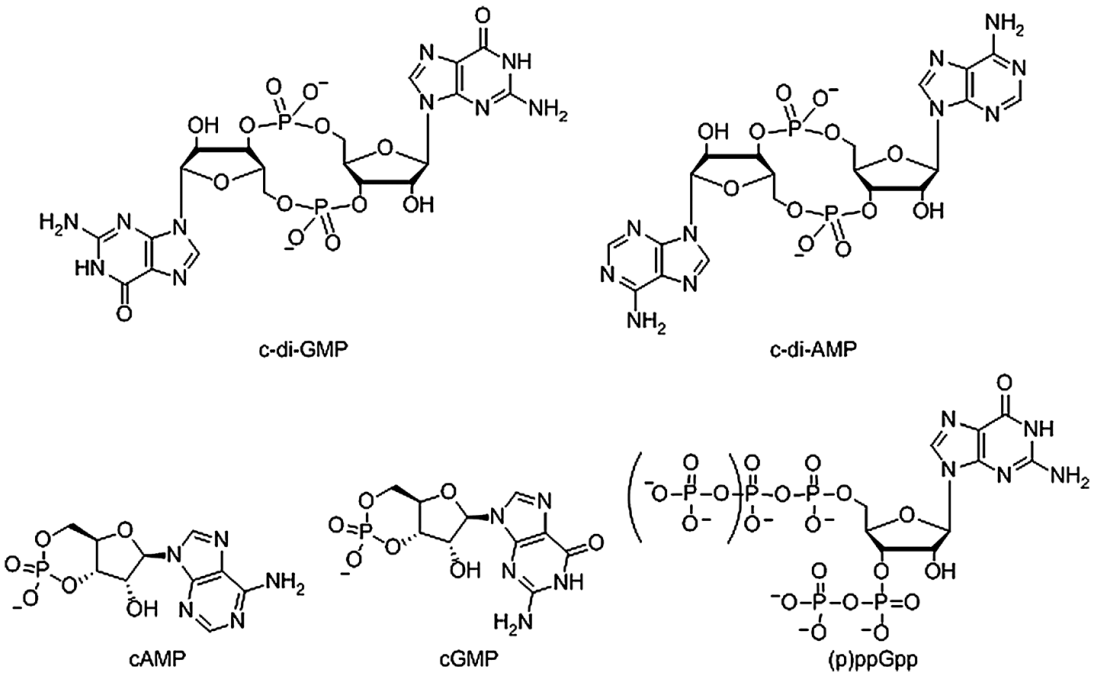


Figure 15. Structure of nucleotide second messengers found in bacteria (Kalia *et al.*, 2013).

The best known example is cyclic AMP (cAMP), which is also the first discovered "second messenger" molecule. In *E. coli* cAMP is involved in catabolite repression by activating catabolic genes in complex with the CRP protein. As a result, cAMP is involved in regulatory mechanisms that lead to the recovery of one's preferred carbon source (Buettner *et al.*, 1973, Gorke & Stulke, 2008). In addition to cAMP and cGMP, the cyclic dinucleotides are other important signaling molecules and become the focus of research in recent years. Above all, c-di-GMP was studied for 25 years in many bacteria and it has been shown that c-di-GMP plays a key role in the change of lifestyle between motility and sessility (Simm *et al.*, 2004). In addition, it is important for biofilm formation, virulence, cell cycle and differentiation through mechanisms such as the production of extracellular polysaccharides, adhesive proteins, pili and flagella (Wolfe & Visick, 2008, Romling,

2008). The formation of c-di-GMP is catalyzed by di-guanylate cyclases, which all have a GGDEF motif in their active sites (Paul *et al.*, 2004). Phosphodiesterases build the c-di-GMP from the linear product pGpG, which is then split into two GTP molecules (Christen *et al.*, 2005, Schmidt *et al.*, 2005).

B.6.1 Cylic diadenosine monophosphate (c-di-AMP)

The investigation of the signaling molecule c-di-AMP is still very actual. The discovery of c-di-AMP happened purely by chance during the study of DNA integrity scanning protein A (DisA) from *B. subtilis* (Bejerano-Sagie *et al.*, 2006). With the aim to investigate this mechanism, DisA was crystallized from *Thermotoga maritima* and interestingly a ligand was present within the crystal structure of the protein, which could be identified as c-di-AMP (Fig. 16). DisA was subsequently confirmed as diadenylate cyclase (DAC). The DAC activity was not affected by linear DNA, but reduced by "Holliday junctions" drastically (Witte *et al.*, 2008). This suggests that the signal transduction for DNA integrity depends on c-di-AMP. The first report of c-di-AMP production in *B. subtilis* cells brought the activity of DisA in relationship with sporulation. When DisA activity was inhibited by DNA damage, the resulting reduced c-di-AMP level leads to a delay in sporulation (Oppenheimer-Shaanan *et al.*, 2011). Thus, the DisA-mediated DNA integrity is not only controlled during sporulation, but probably in any stage of *B. subtilis* growth. DACs catalyze the condensation of two molecules of ATP through 3'-5 'phosphodiester to the c-di-AMP (Witte *et al.*, 2008).

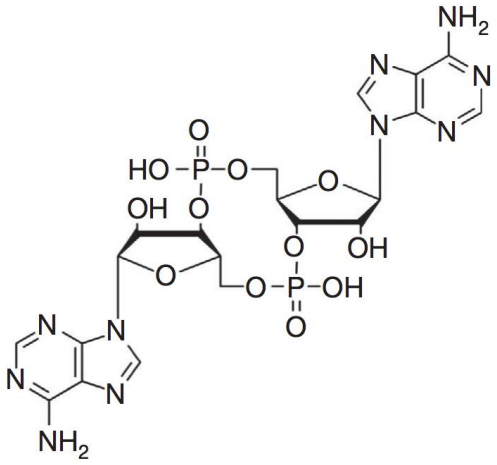


Figure 16. Cyclic di-adenosine monophosphate

c-di-AMP is condensed from two molecules of AMP on the 3'-5'-phosphodiester (Kalia *et al.*, 2013)

While, these effects of c-di-AMP signaling have not been extensively studied, these data indicate that this pathway could be a potential target for antimicrobial and anti-biofilm therapy (Chaudhuri *et al.*, 2009). In *S. aureus*, c-di-AMP has a role in resistance to extreme cell wall stress. In addition, high levels of c-di-AMP concentration are associated with increased resistance to some cell wall-active antimicrobials in *S. aureus* and *B. subtilis*. In addition, there are important findings that c-di-AMP signaling stimulates biofilm formation (Corrigan *et al.*, 2011, Luo & Helmann, 2012).

B.7 The aim of study: What is the role of putative *PII-like* in *S. aureus*?

In this study, we aimed to elucidate the presence of *PII-like* protein in *S. aureus* NCTC8325 strain HG003. Initially, the key idea of the project was to investigate the role of *PII-like* protein in nitrogen regulation of *S. aureus* and characterize its functions biochemical and physiological. For this purpose we aimed,

I. Creation of a *PII-like* (SAOUHSC_00452) deletion mutant (Δ*PII-like*) in *S. aureus* NCTC8325 by homologous recombination and,
II. Comparative phenotypic characterization of Δ*PII-like* with wild type strain HG003

As mentioned before, during our studies Corrigan et *al.* (2013) published the initially results about *PII-like*. They have done the first annotation of *PII-like* in *S. aureus* (MRSA) by using a genome-wide nucleotide protein interaction screen (DRaCALA) and renamed as PstA (*PII-like* signal transduction protein A) (Corrigan *et al.*, 2013). They identified PstA as a c-di-AMP binding protein and suggested that PstA play crucial role in various cellular processes by interacting of other cellular proteins or ligands.

During our studies we couldn't observe any significant phenotype, except growth delay/defect during the exponential phase, which was caused by the absence of *PII-like*. Therefore, we focused on the interaction characteristics of *PII-like*. We aimed to investigate the interaction partners of *PII-like* using ITC and Biacore surface plasmon resonance and planned to test several molecules such as c-di-AMP, cAMP, ATP, ADP, AMP, GTP and TTP.

C. MATERIAL and METHODS

This section was intended as a supplement to the **'Material and Methods'** of **Chapter-I**.

C.1 Materials

C.1.1 Chemicals

Tab. C.1 Used chemicals and their source of supply

Chemicals	Supplier
4-aminobenzoic acid	Sigma-Aldr., Deisenhofen
Adenosine 3′,5′-cyclic monophosphate (cAMP)	Sigma-Aldr., Deisenhofen
Adenosine 5′-monophosphate sodium salt	Sigma-Aldr., Deisenhofen
Adenosine 5′-diphosphate sodium salt	Sigma-Aldr., Deisenhofen
Adenosine 5′-triphosphate disodium salt hydrate	Sigma-Aldr., Deisenhofen
Ammoniumchlorid	Merck, Darmstadt
Anhydrotetracycline	Sigma-Aldr., Deisenhofen
Benzyl viologen	Sigma-Aldr., Deisenhofen
Boric acid	Sigma-Aldr., Deisenhofen
Casamino acids	Carl Roth, Karlsruhe
Citric acid	Sigma-Aldr., Deisenhofen
Coomassie Brilliantblau R-250	AppliChem GmbH, Darmstadt
Cobalt (II) chloride	Sigma-Aldr., Deisenhofen
Cupper (II) chloride	Sigma-Aldr., Deisenhofen
Cyclic diadenosine monophosphate	BIOLOG, Bremen
Disodium hydrogen phosphate	Merck, Darmstadt
Dithionite	Sigma-Aldr., Deisenhofen
D-pantothenic acid (Ca salt)	Sigma-Aldr., Deisenhofen
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldr., Deisenhofen
Guanosine 5′-triphosphate sodium salt hydrate	Sigma-Aldr., Deisenhofen
Iron (II) chloride	Sigma-Aldr., Deisenhofen
Mangan (II) chloride	Sigma-Aldr., Deisenhofen
Mineral oil	Sigma-Aldr., Deisenhofen
MOPS	Merck, Darmstadt
MOPS	Merck, Darmstadt

Chemicals	Supplier
$NaNO_3$	Merck, Darmstadt
Nickel (II) chloride	Sigma-Aldr., Deisenhofen
Nicotinic acid	Sigma-Aldr., Deisenhofen
Oxyrase	Oxyrase, Mansfield, Ohio
Potassium chloride	Merck, Darmstadt
Potassium phosphate	Merck, Darmstadt
Riboflavin	Serva, Heidelberg
Safranin	Sigma-Aldr., Deisenhofen
Thiamine dichloride	Sigma-Aldr., Deisenhofen
Thymidine 5′-triphosphate sodium salt	Sigma-Aldr., Deisenhofen
Triton X-100	Sigma-Aldr., Deisenhofen
Tween-20	Sigma-Aldr., Deisenhofen
Zinc chloride	Sigma-Aldr., Deisenhofen
β-Mercaptoethanol	Merck, Darmstadt
12% SDS-PAGE precast gels	Biozol (Expedeon), Eching

C.1.2 Molecular biological Kits

Tab. C.2 Used molecular biological kits and their source of supply

Supplier	Kit
QIAGEN GmbH, Hilden	QIAquick-Gel extraction Kit QIAGEN-Plasmid-Midi-Kit QIAGEN-Plasmid-Mini-Kit QIAquick PCR Purification Kit
Roche, Mannheim	Rapid DNA Dephos & Ligation Kit
Stratagene GmbH, Heidelberg	StrataClean®-Resin

C.1.3 Columns and buffers used in chromatography

Tab. C.3 ÄKTA and affinity chromatography: used columns, buffers and their source of supply

Supplier	Column material	Buffer compositions
IBA GmBH, Göttingen	*Strep* Tactin Superflow *Strep*-Tactin® Superflow® Cartridge H-PR	Buffer W (washing buffer, 10x) 1 M Tris-Cl pH 8.0, 1.5 M NaCl 10 mM EDTA Buffer E (elution buffer, 10x) 1 M Tris-Cl pH 8.0, 1.5 M NaCl 10 mM EDTA, 25 mM desthiobiotin Buffer R *(*regeneration buffer, 10x) 1 M Tris-Cl pH 8.0, 1.5 M NaCl, 10 mM EDTA, 10 mM HABA (hydroxy-azophenyl-benzoic acid)

C.1.4 Molecular weight markers

Tab. C.4 Used molecular weight markers, loading dyes and their source of supply

Supplier	Product
Fermentas, St. Leon-Rot	GeneRulerTM 1 kb DNA ladder GeneRulerTM DNA ladder Mix PageRulerTM prestained protein ladder

C.1.5 Consumable materials and plastic articles

Tab. C.5 Used consumable materials, plastic articles and their source of supply

Supplier	Product
GE Healthcare Europe, München (Whatman, Dassel)	Whatman® Protran® nitrocellulose, filter sterilizer (0.2 µm)
Greiner Bio-One, Frickenhausen	FalconTM tube PS 12 ml, FalconTM tube PS 50 ml, single-use inoculating loop
Henke Sass Wolf, Tuttlingen	Injection Norm-Ject (1 ml, 5 ml, 20 ml)
Millipore, Schwalbach	Filter Millipak Express 20 (0.22 µm), StericupTM PES membrane (0.22 µm)
Molecular Bioproducts, San Diego	200 µl reaction tubes
Roth, Karlsruhe	glass beads (0.25-0.5 mm)
Thermo Fisher Scientific	96 well plate NunclonTM surface 96 well plate Nunc black U96 PP-0.5 ml
Spectrum, Frankfurt	Dialysis device Float-A-Lyzer G2

C.1.6 Computer programs

Tab. C.6 Used computer programs and their source of supply

Supplier / Source of Reference	Product
KEGG (http://www.genome.jp/kegg/)	Kyoto Encyc. of Genes and Genomes
NCBI, Bethesda (http://blast.ncbi.nlm.nih.gov/Blast.cgi)	Basic local alignment search tool (BLAST)
Invitrogen, Karlsruhe	VectorNTI Suite 10.3
Scientific-Educational Software, USA	Clone Manager Professional 9
DNASTAR, Inc., USA	SeqMan NGen
Microsoft Dtld. GmbH, Unterschleißheim	Microsoft Office 2010/2013
Thomson Reuters, Carlsbad	Endnote X4, Endnote X7

C.1.7 Special machines and devices used in the laboratory

Tab. C.7 Special machines and their source of supply

Device	Model	Supplier
Incubation chamber	B5042E	Heraeus Holding GmbH, Hanau
Bunsenburner	Phoenix II eco	Schuett-biotec GmbH, Göttingen
Ice supply	AF 100	Scotsman Ice Systems, Mailand
Elektroporator	Multiporator®	Eppendorf AG, Hamburg
Gel documentation system	-	LTF Labortechnik GmbH & Co. KG, Wasserburg/Bodensee
Gel documentation system	-	Vilber Lourmat, Eberhardzell
Mighty-small (SDS gel electrophoresis chamber)	-	Hoefer Scientific, San Francisco
Heating block	-	Bachofer Laboratoriumsgeräte, Reutlingen
Microwave	NN-SD456W	Panasonic Corporation, Osaka
Magnet stirrer	-	RCT basics IKA®-Werke GmbH & CO. KG, Staufen IKA RCT IKAMAG
PCR-Cycler	Primus 96 plus	MWG-Biotech AG, Penzberg

Device	Model	Supplier
pH-Meter	Hydrus 300	Fisher Scientific UK, Loughborough
Photometer	Helios alpha	Thermo Fisher Scientific Inc. Waltham
Plate Reader	Infinite M200	Tecan Group Ltd., Männedorf
Ultrapurewater apparatus Milli-Q	Plus PF	EMD Millipore Corporation, Billerica
Power supply	LKB-GPS 200/400 2297 Macrodrive 5	Pharmacia, Ratingen LKB Bromma
Laminar Workflow	SterilGARD VBM 400	Baker Company Inc., Sanford
Drying incubator	T 6120	Heraeus Holding GmbH, Hanau
Vortex	Vortex Genie 2	Bender & Hobein GmbH, Zürich
Scale	Weighing machine BP 61-OCE special accuracy weighing machine Kern EW 4200-2NM	Sartorius AG, Göttingen KERN & SOHN GmbH, Balingen
Waterbath		Bachofer Laboratoriumsgeräte, Reutlingen
Centrifuges	Centrifuge Z 216 MK Centrifuge Multifuge X3R Table centrifuge Z 233 M-2 Vakuumzentrifuge Univapo 100 H	Hermle Labortechnik, Wehingen Thermo Scientific, Karlsruhe Hermle Labortechnik, Wehingen UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg
FastPrep FP120	- Bio101	Thermo Savant
French pressure cell press	-	Thermo Scientific, Waltham, MA, USA
ÄKTA *Purifier*, ÄKTA *Explorer*	-	GE Healthcare Europe, München (Amersham Biosciences, Freiburg)
SpectraMax 340 microtiter plate spectrophotometer	-	Molecular Devices, Sunnyvale

C.1.8 Bacterial strains

Tab. C.8 Used bacterial strains

Strain/ Plasmid	Characteristics / Genotype	Source of Reference
Staphylococcus carnosus TM300	SK311	(Schleifer & Fischer, 1982)
Escherichia coli DH5α	*endA1 hsdR17* (rK- mK+) *supE44 thi-1recA1 gyrA* (Nalr) *relA1* Δ(*lacZYAargF*) *U169* *deoR* (Φ80 *dlacZ*ΔM15)	(Hanahan, 1983)
pBluescript® II	KS (+)	
Staphylococcus aureus RN4220	Derivative of NCTC8325-4. r- m-	(Herbert *et al.*, 2010)
HG003	Derivative of NCTC8325-4. r- m-	(Iordanescu & Surdeanu, 1976)

C.1.9 Plasmids

Tab. C.9 Constructed plasmids

Plasmid	Size (bp)	Inducer	Host	Selection
pBT2Δ*PII-like_erm*B	11287	- -	*E. coli* *S. aureus* RN4220 *S. aureus* HG003	Am^R (100 µg/ml) Cm^R (10 µg/ml) Em^R (2,5 µg/ml) Cm^R (10 µg/ml) Em^R (2,5 µg/ml)
pTX30*PII-like*	7274	D-(+)-Xylose (0,5 %) D-(+)-Xylose (0,5 %)	*S. aureus* RN4220 *S. aureus* HG003	Tet^R (25 µg/ml) Tet^R (25 µg/ml)
pASK-IBA3-*PII-like*–Strep tag II	3475	ATc (200µg/L)	*E. coli*	Am^R (100 µg/ml)

C.1.10 Oligonucleotides

Synthetic oligonucleotides were ordered through Biomers (Ulm, Germany).

Tab. C.10 Oligonucleotides for cloning, PCR identification and verification

Primer	**Sequence (5'→3')**	**REN**	**Purpose of use**
Fwd_upstream_PII_*Eco*RI	GGATCGAATTCCCTGTACTCATTGACGAAGCACAC	*Eco*RI	Δ*PII-like* upstream
Rev_upstream_PII_*Sma*I	GGATCCCCGGGCTATCTTGATCTTGTACGATCGCT	*Sma*I	Δ*PII-like* upstream
fwd_downstream_PII_SmaI	GGATCCCCGGGTGATGCATTCCATCAATTTTAATTCT	*Sma*I	Δ*PII-like* downstream
Rev_downstream_PII_BamHI	GGATCGGATCCTCAATACGATCATCCGCCGTAA	*Bam*HI	Δ*PII-like* downstream
fwd_*erm*B_PII	GGATCACCTCTTGAAAATGTTGTTGAAG	-	*erm*B cassette
Rev_*erm*B_PII	GATCAGTCAAGCAACCCAAAATTGTTA	-	*erm*B cassette
Comp_Fwd_PII_*Bam*HI	GGATCGGATCCGTTGAAGACACGTATCAAACTATCA	*Bam*HI	complementation
Comp_Rev_PII_*Xma*I	GGATCCCCGGGCGCAGTCAAGCAACCCAAAATT	*Xma*I	complementation

Tab. C.11 Oligonucleotides for DNA sequencing and verification

Primer	**Sequence (5'→3')**	**Purpose of use**
pBT2a	CCAGGCGTTTAAGGGCACCA	sequence verification
pBT2b	CACGGTGCCTGACTGCGTTAGCA	sequence verification
fwd_*erm*B_PII	GGATCACCTCTTGAAAATGTTGTTGAAG	sequence verification
Rev_*erm*B_PII	GATCAGTCAAGCAACCCAAAATTGTTA	sequence verification
Fwd_Mutant_Sp_start-up_ΔPII	TGAGCCGCATTGAGTGTGAA	sequence verification
Rev_Mutant_Sp_end-down_ΔPII	ACTAAATGCACCGGACGTCT	sequence verification
fwd_end-PII-up_seq	GGATCTGTATTAAAGGTCATACCAGCTT	sequence verification
rev_start-PII-down_seq	GGATCGTTTCACATTGACTATCTGTTTG	sequence verification
pASK-IBA_fwd	GAGTTATTTTACCACTCCCT	sequence verification
pASK-IBA_rev	CGCAGTAGCGGTAAACG	sequence verification

C.1.11 Media for cultivation

The culture media were autoclaved for 20 min at 121 °C and 2 bar. For the preparation of plates 15 g agar were added per liter. Some components were sterile filtered and added to media.

Tab. C.12. Media for cultivation

Culture medium	**Composition (in 1 L)**
B2	10 g Casein hydrolysate, 25 g Yeast extract, 5 g Glucose, 25 g NaCl, 1 g K_2HPO_4
B3	2 g Casein hydrolysate, 5 g Yeast extract, 1 g Glucose, 15 g NaCl, 1 g K_2HPO_4
Luria Bertani (LB)	10 g Trypton, 5 g Yeast extract, 10 g NaCl (pH: 7.0)
Nutrient broth (NB)	5 g Pepton from meat, 3 gr meat extract (pH: 7.0)
Synthetic medium (modified)	10 mM Na_2HPO_4, 10 mM KH_2PO_4, 9.34 mM NH_4Cl, 8.55 mM NaCl, 0.81 mM $MgSO_4$, 0.142 mM citric acid, 40 mM MOPS pH 7.0, and 7.5 mM glucose (or 15 mM glycerol). 20% (w/v) casamino acids Vitamins (0.29 µM 4-aminobenzoic acid, 0.04 µM biotin, 0.81 µM nicotinic acid, 0.21 µM D-pantothenic acid (Ca salt), 0.29 µM thiamine dichloride and 0.26 µM riboflavin, and Trace elements 7.5 µM $FeCl_2$, 0.51 µM $ZnCl_2$, 0.5 µM $MnCl_2$, 0.097 µM boric acid, 1.46 µM $CoCl_2$, 0.015 µM $CuCl_2$ and 0.1 µM $NiCl_2$
Tryptic soy broth (TSB)	30 g Tryptic soy broth powder

C.2 METHODS

This section was intended as a supplement to the **'Material and Methods'** of **Chapter-I**.

C.2.1 Determination of cell density for growth behavior

Bacterial growth was monitored by determination of the optical density. A preculture was inoculated from a fresh agar plate culture in corresponding medium and cultured overnight at 37 °C on a shaker. Depending on the selection marker, corresponding antibiotics were added to all pre-cultures with respective final concentrations. From this pre-culture, 50 ml of TSB was inoculated to an OD_{578} 0.07, and cultured at 37 °C with shaking (150 rpm). All antibiotics were added to respective final concentrations. The optical density at 578 nm was determined every hour.

C.2.2 Cell disruption using *French press*

A French pressure cell is an apparatus with which bacterial cells can be lysed using high pressure. The cell suspension was poured into a pressure cell into which a piston is inserted. The piston is slowly pushed further into the cylinder by a hydraulic press which leads to a high pressure inside the cylinder. The pressure is released by opening a needle valve, causing the bacterial cell walls and membranes to disrupt upon exit.
Frozen cell pellets were resuspended (vortexed) in 25 ml lysis buffer (20 mM Tris-HCl pH 7.8) containing a protease inhibitor cocktail and transferred to a pre-assembled French press cell with a 1" piston. Two independent French press runs were done at the *high* setting with a pressure setting of 900. The French press cell was chilled before use and samples were kept on ice to reduce protein degradation.

C.3. Working with proteins

C.3.1 Determination of protein concentration (Bradford assay)

Total protein concentrations were determined by the method of Bradford (Bradford, 1976). Here were more defined concentrations of BSA as a standard. The optical density was measured at a wavelength of 595 nm (OD_{595}). A calibration series with different BSA concentrations (0-100 µg) was created in the corresponding sample buffer, to quantify the respective Bradford assay. According to the estimated standard curve, the protein concentration of the sample could be calculated with the linear equation y=mx+b. In

addition, the protein concentration of the proteins of a cell extract was estimated by SDS-PAGE according to a defined amount of purified protein.

C.3.2 Gel electrophoretic separation of proteins using SDS-PAGE

The electrophoretic separations of proteins were performed using commercially available 12% precast gels (Biozol (Expedeon), Eching). For electrophoresis gels were inserted into a chamber and filled up with RunBlue SDS Running buffer (Biozol (Expedeon), Eching). Protein containing samples were boiled in 3x Laemmli sample buffer (Laemmli, 1970) for 10 min at 90 °C prior to the gel electrophoresis. Gels were started with 30 mA for accumulation of proteins in the stacking gel, before the current was set to 90 mA until the run was stopped.

C.3.3 Staining and destaining of SDS-PAGEs

After gel electrophoresis, gels were incubated in Coomassie brilliant blue-staining solution for several hours. Incubation was carried out until the gel is stained blue. Next, the Coomassie blue was removed from the gel using 10% acetic acid (destaining solution) for 1-2h or overnight at RT on a lateral buckling shaker. The decolorized gel stored in distilled water at room temperature.

C.3.4 Overexpression of *PII-like*_Strep-tag II in *E. coli* BL21 (DE3)

A synthetic *S. aureus PII-like* gene fused with a Strep tag II sequence at the C-terminus was cloned into the anhydrotetracycline (ATc) inducible pASK-IBA3 vector. Overexpression of the recombinant *PII-like* protein was performed in *E. coli* BL21 (DE3) and recombinant protein was affinity purified on a Strep-tactin superflow column according to manufacturer's protocol.

A 10 ml over day culture was inoculated from a single transformation colony (*E. coli* BL21 (DE3) carrying pASK-IBA3_*PII-like*_Strep II expression construct) in LB media with corresponding antibiotics (Ampicillin 100 µg/ml). An appropriate volume (calculated to a start OD_{578} 0.1) of this culture was used to inoculate a 1 L overnight culture. After incubation at 37°C, and reaching an optical density between 0.6-0.8, cells were induced with ATc (200 µg/L) and incubated overnight. Same cultivation steps were done without induction. After incubation at 37°C overnight, 1 L of cultures with/without induction were harvested by 30 min centrifugation at 4°C and 5000 rpm. The pellet was

washed by resuspending the cells in 25 ml cold phosphate buffer (20 mM), vortexed and centrifuged for 30 min at 5,000 rpm and 4°C. The supernatant was decarded and the pellet stored at −20°C until French press treatment. The uninduced and induced samples were analyzed on a SDS-PAGE gel.

C.3.5 Purification of *PII-like* using Twin-Strep-tag affinity chromatography

(IBA GmBH, Göttingen)

The *Strep*-tag purification system is based on the highly selective binding of engineered streptavidin, called *Strep*-Tactin, to *Strep*-tag II fusion proteins. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its biological activity. *Strep*-tag/*Strep*-Tactin affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. Because of its small size, *Strep*-tags generally does not interfere with the biological activity of the fusion partner. The Twin-Strep-tag is a dimeric version of the Strep-tag II and therefore binds with the same selectivity to Strep-Tactin but with a higher affinity. This higher affinity allows the purification of Twin-Strep-tagged proteins even from batch or cell culture supernatants with good yields. In addition, the Twin-Strep-tag tolerates higher amounts of detergents and salts in buffers compared to Strep-tag II. The Strep-tag II is an eight-residue minimal peptide sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) that exhibits intrinsic affinity toward streptavidin and can be fused to recombinant proteins in various fashions.

The lysate of French press treated cells was thawed and centrifuged for 20 min at 5,000 rpm and 4°C to separate soluble and insoluble cell components. To start the *Strep*-tag purification steps, the *Strep* Tactin Superflow column was first equilibrated with two column volumes (2 ml) Buffer-W. After equilibration, 0.5-10 ml clean extract of *PII-like* was added to the column. Then, the column was washed five times with one ml Buffer-W, after the cell extract has completely entered. To elute *PII-like* protein, 500 µl Buffer-E was added six times to the column and eluted samples were collected separately for SDS-PAGE analysis. The eluted samples were stored at 4 °C until the analysis step. In regeneration step, column was regenerated by adding Buffer-R until a color change was observed. The color change from yellow to red indicates the regeneration process. The

intensity of the red color is an indicator for the column activity status for the next round of purification. Buffer-R was removed from the column by the addition of Buffer-W. To assess the purification efficiency, the flow-through, washing and elution-fractions were analyzed in a SDS-PAGE. Fractions containing the desired protein were pooled and subjected to dialysis. Additionally, purified *PII-like* was mixed with 15% glycerol at -20 °C for long time storage.

C.3.6 Purification of *PII-like*_Strep-tag II using ÄKTA

An ÄKTA Explorer FPLC system from Amersham Biosciences (Freiburg, Germany) was used for large amount of protein purification. Protein purification was carried out at 4 °C to 8 °C. All purification steps were performed according to the protocol of Twin-Strep-tag affinity chromatography (see section C.3.5).

C.3.7 Dialysis

The collected elution fractions were transferred to pre-soaked dialysis tubes with a molecular weight cut-off (MWCO) of 0.5 kDa, sealed and incubated in 1 L of cold dialysis buffer overnight at 4°C. The next day the dialysis buffer was exchanged, dialysis carried out for additional 5-7 h and the concentration of the protein was measured (Bradford and Nanodrop).

C.3.8 Measurement of protein concentrations

The amino acids tyrosine, tryptophane and cystine (not cystein) absorb light at 280 nm and can be used to calculate the protein concentration if the sequence is known (Gill & von Hippel, 1989). To determine the extinction coefficient of proteins, the ProtParam tool from the ExPASy Bioinformatic Resource Portal (Wilkins *et al.*, 1999) was used. It relies on the following formula (Equation C-4):

$$Extinction\ coefficient = num(Tyr) \cdot 1490 + \mathrm{num(Trp)} \cdot 5500 + \mathrm{num(Cystine)} \cdot 125$$

To calculate the protein concentration from the E_{280} nm value and extinction coefficient the following formula (Equation C-5) was used:

$$E_{280nm} = \frac{Extinction\ coefficient}{Molecular\ weigth}$$

The NanoDrop system was blanked with dialysis buffer, the E280 value measured and the concentration calculated with Equation C-5 (Schuster, 2011).

C.4 Microtiter plate adherence assay (Biofilm formation)

To determine the biofilm formation, cells were grown overnight in TSB medium. Cultures were then diluted to an OD_{578} of 0.07 in fresh TSB medium, and 200 µl of the cell suspension per well was used to inoculate sterile, flat-bottom 96-well polystyrene microtiter plates. After incubation for 24 h at 37°C without shaking, the wells were washed twice with phosphate buffered saline (pH 7.2) and dried in an inverted position, and adherent cells were stained with 0.1% safranin. The absorbance of stained biofilms was measured at 450 nm, using a microtiter plate reader (SpectraMax 340) (Schlag *et al.*, 2007).

C.5. Nitrate reductase activity

Nitrate reductase assay was performed by the method of Lowe and Evans (Lowe & Evans, 1964) with modifications of Neubauer et *al.* (Neubauer & Götz, 1996). *S. aureus* (WT) and Δ*PII-like* cells were grown in Falcon tubes with BM (without shaking). Oxyrase and mineral oil were added to generate anoxic conditions. Cells were harvested in the mid-exponential growth phase and washed twice with potassium phosphate buffer (100 mM, pH 7.2). The assay mixture (total volume, 3 ml) was composed of potassium phosphate buffer (90 mM, pH 7.2), containing benzyl viologen (1 mM) as an artificial electron donor, and the reducing agent dithionite (4.7 mM). The addition of dithionite in excess facilitated handling without an anaerobic chamber. Cells were added to a final OD_{578} of 0.4, and the mixture was stirred at 37°C. Subsequently, the reaction was started by the addition of 10 mM sodium nitrate. Samples were taken at various time intervals up to two min and nitrate reduction was stopped by vigorous vortexing the mixture to oxidize all dithionite and benzyl viologen. After removal of the cells by centrifugation, the nitrite concentrations in the samples were measured (Neubauer & Götz, 1996).

C.5.1 Detection of nitrite

The nitrite concentration was determined colorimetrically by the method of Nicholas and Nason (Nicholas & Nason, 1957) as modified by Showe and DeMoss (Showe & DeMoss, 1968). The reaction is converted to nitrite, a red azo compound. The reactive compound

in this case is the nitrosyl-ion NO^+, which is present in solutions of the nitrous acid in equilibrium and reacts with primary amines (sulfonamide) into a diazonium ion (here, N-naphthyl-ethylene diamine). This reaction in a colored azo compound which can be detected photometrically at 540 nm (Neubauer & Götz, 1996).

C.6 Ligand binding

C.6.1 Isothermal titration calorimetry (ITC)

Isothermal Titration Calorimetry (ITC) experiments were performed on a VP-ITC (MicroCal, LCC) instrument. The protein solution containing Strep II-tagged *PII-like* was exhaustively dialyzed against 20 mM Tris-HCl, 150 mM NaCl (pH 7.5) prior to ITC measurements, and tested ligands (ATP, ADP, AMP, GTP, TTP, c-di-AMP, cAMP) were individually dissolved into the same buffer. In a typical setup, *PII-like* (100 µM) was placed in the *sample cell* and the ligand (different concentrations in the same buffer) in the *titration syringe*. All titrations were done at 24 °C with a reference power of 15 (µCal/sec.) and a stirring speed of 155 rpm. Data analysis was carried out using the MicroCal ORIGIN software (Northampton, MA).

C.6.2 Biacore surface plasmon resonance detection

Surface plasmon resonance experiments were performed using the Biacore X100 biosensor system (GE Healthcare/Biacore AB, Uppsala, Sweden). *S. aureus PII-like* was coupled to a CM-5 chip *via* amine coupling chemistry. To increase the sensitivity of the nucleotide binding we saturated the chip surface with *PII-like*. Kinetic data were recorded for injections of all nucleotides in running buffer 20 mM Tris-HCl, 150 mM NaCl, % 0.05 Tween, pH 7.5. Data from an empty flow cell (FC1) were subtracted as reference to correct for unspecific binding of the analytes to the chip surface. Data were analyzed using the Biacore X100 Evaluation software.

D. RESULTS

D.1 Generation of a Δ*PII-like* mutant in *S. aureus* NCTC8325 strain HG003

In *S. aureus* NCTC8325 strain HG003, putative *PII-like* gene (SAOUHSC_00452) is located as a member of an operon with 330 bp nucleotides in size (Fig. 17.). We used the homologous recombination method (Brückner, 1997) for the deletion of *PII-like*. The method is based on the exchange of genes in the genome of *S. aureus.*

SAOUHSC_00450: Orn/Lys/Arg decarboxylase enzyme
SAOUHSC_00451: thymidylate kinase (tmk) with metabolism of nucleotides and nucleic acids
SAOUHSC_00452: hypothetical protein (putative P_{II}-like protein)
SAOUHSC_00453: hypothetical protein
SAOUHSC_00454: DNA polymerase III subunit delta with purine-pyrimidine metabolism, Metabolic pathways, DNA replication, mismatch repair and homologous recombination

Figure 17. Genomic context of *PII-like*

PII-like is an operon member and located upstream thymidylate kinase (TMK) which is responsible for nucleic acid metabolism.

The corresponding putative *PII-like* gene was deleted partially (Fig. 18.) and 5 'and 3' parts of putative *PII-like* were left to avoid any polar effects. Therefore, the shuttle vector pBT2 was used, which contains a temperature sensitive replicon for staphylococci. No plasmid replication takes place, when cultivated above 30°C. As selection marker, the erythromycin resistance cassette derived from plasmid pEC2 was used and the inactivation vector was cloned in *E. coli* DH5α (Brückner, 1997). To generate a Δ*PII-like* mutant, an allelic replacement shuttle vector pBT2Δ*PII-like_ermB* (Fig. 19.) was constructed, which contained an expression cassette of the erythromycin resistance gene (*erm*B) flanked by two DNA fragments from the flanking region of the *PII-like* gene, and transformed to *S. aureus* by electroporation.

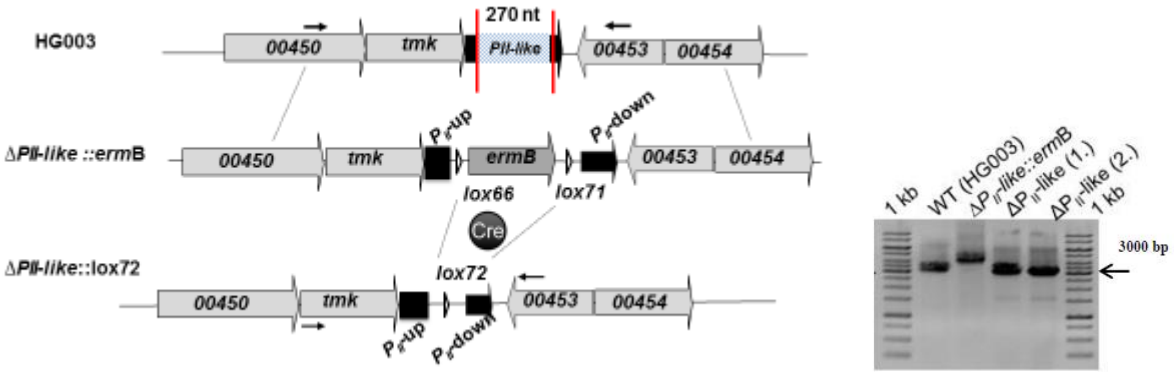


Figure 18. Deletion of putative *PII-like* gene in *S. aureus* HG003 by homologous recombination

The 5 'and 3' parts of putative PII-like gene was left to avoid any polar effects. The resistance marker was removed by *Cre*-recombinase (expressed by pRAB1 plasmid), which recognized the corresponding *lox66*/*71* sites leaving the site *lox72* in the chromosome, and a marker-less mutant was generated.

Approximately 200-300 erythromycin- and chloramphenicol- resistant colonies were selected, in which the existence of the allelic replacement vector pBT2Δ*PII-like_ermB* was confirmed by plasmid re-isolation, *ermB*-PCR amplification, control digestions and sequencing.

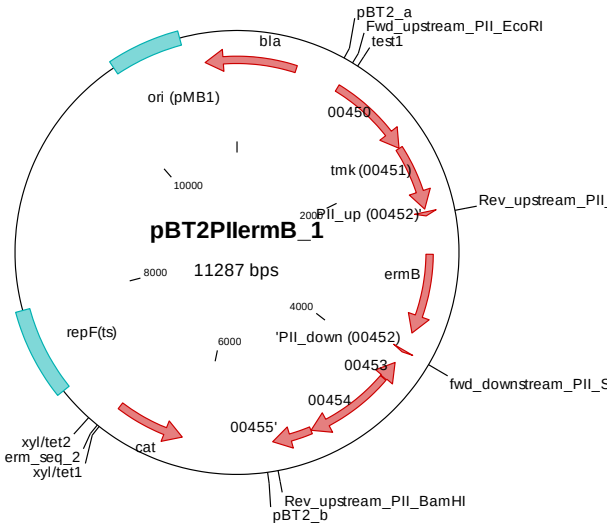


Figure 19. Construct of knock-out vector pBT2Δ*PII-like_ermB*

The temperature sensitive shuttle vector pBT2 was used for deletion of *PII-like* by homologous recombination. The upstream and downstream regions of *PII-like* gene were cloned with an *ermB* cassette.

The candidates showing Em resistance and Cm sensitivity were selected and the colonies only carrying *erm*B cassette with *lox66*/*71* sites on the chromosomal DNA were confirmed by PCR and sequencing (Fig. 18.). After transformation of pRAB1 vector into *S. aureus* Δ*PII-like*::*erm*B cells, the resistance marker was removed by *Cre*-recombinase which recognized the corresponding *lox66*/*71* sites leaving the *lox72* site in the chromosome (Leibig *et al.*, 2008). pRAB1 was removed with temperature up-shift steps from the cells. Some Δ*PII-like* mutant candidates without any antibiotic resistance were selected and verified by sequencing.

D.2 Physiological characterization of Δ*PII-like* mutant

D.2.1 Growth properties of Δ*PII-like* in complex and synthetic media

After generation of a Δ*PII-like* mutant in *S. aureus* HG003, growth analysis were performed using complex and minimal-synthetic media. Growth behavior of WT and Δ*PII-like* mutant were investigated and compared in TSB, LB, NB, B2 and B3 rich media. The growth of the cultures were monitored by determination of the optical density every hour, by measuring the optical density at 578 nm and growth was analyzed until the end of stationary phase.

The growth of cultures were performed under aerobically conditions and shaking by 150 rpm at 37 °C. The components of casein peptone (pancreatic), soya peptone (papain digest.), yeast/beef extract, peptic digest of animal tissue and tryptone in complex media were used as nitrogen sources. Deletion of the putative *PII-like* gene resulted in severe impairment during the exponential phase of growth in nitrogen excess rich mediums (Fig. 20. a.-e.) Unfortunately, no significant difference was observed when the cells reached the stationary phase. Cultures grew to very high optical densities (~ OD_{578}: 25) when complex medium B2 was used. B2 was modified to B3 medium and used as a new medium with reduced components. Also, similar growth properties were observed in B3 medium.

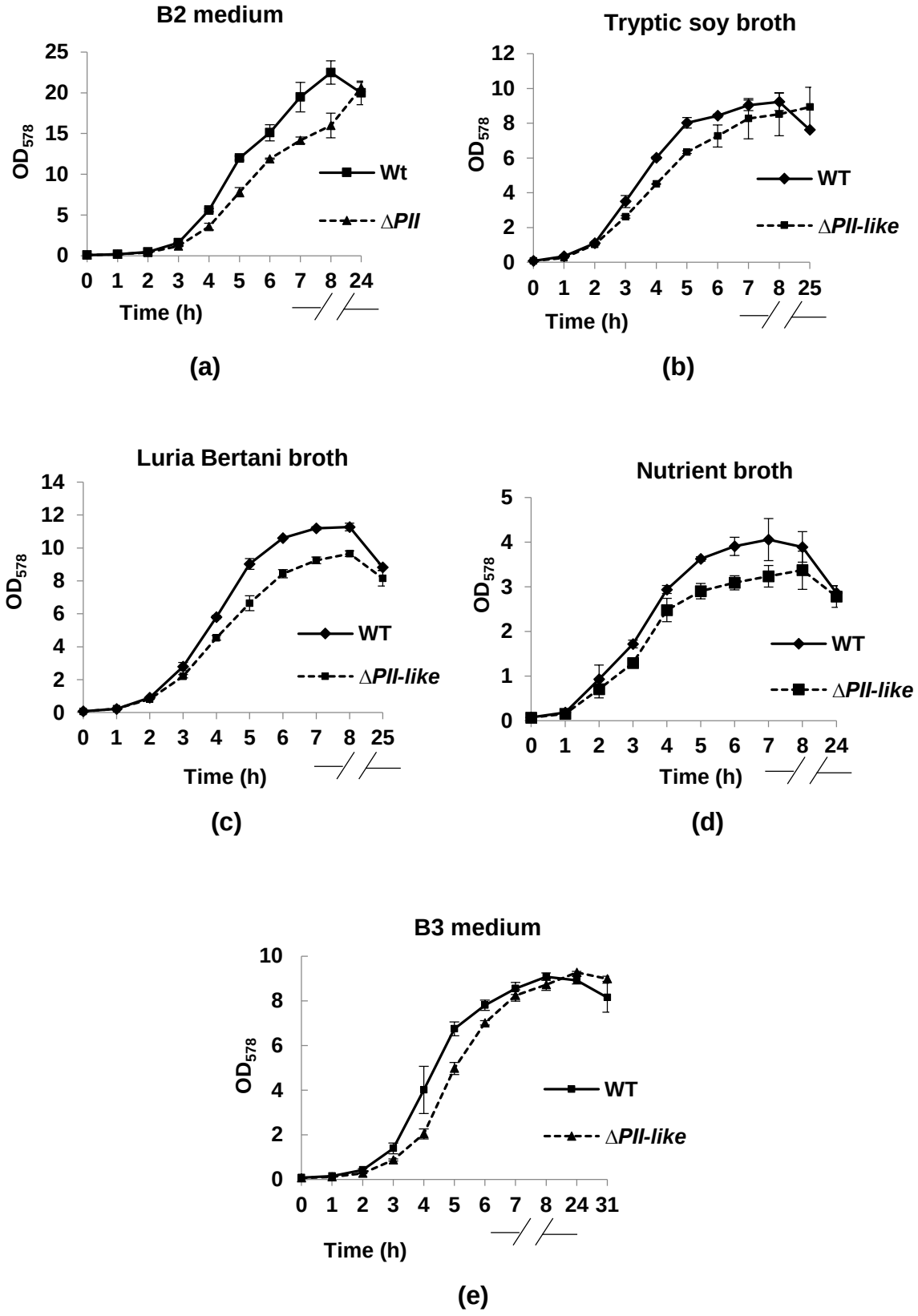


Figure 20. Comparison of growth properties in different complex media

After several repeats of growth experiments with complex media, the Δ*PII-like* mutant showed a slower growth in the exponential phase compared to the WT strain.

D.2.1.2 Growth in minimal medium

To investigate the growth properties of the Δ*PII-like* mutant under nitrogen deficient conditions, a minimal medium was used which was named as *synthetical medium* (Gertz *et al.*, 1999). *S. aureus* and Δ*PII-like* mutant strains were grown in a modified synthetic medium (Tab. C.12).

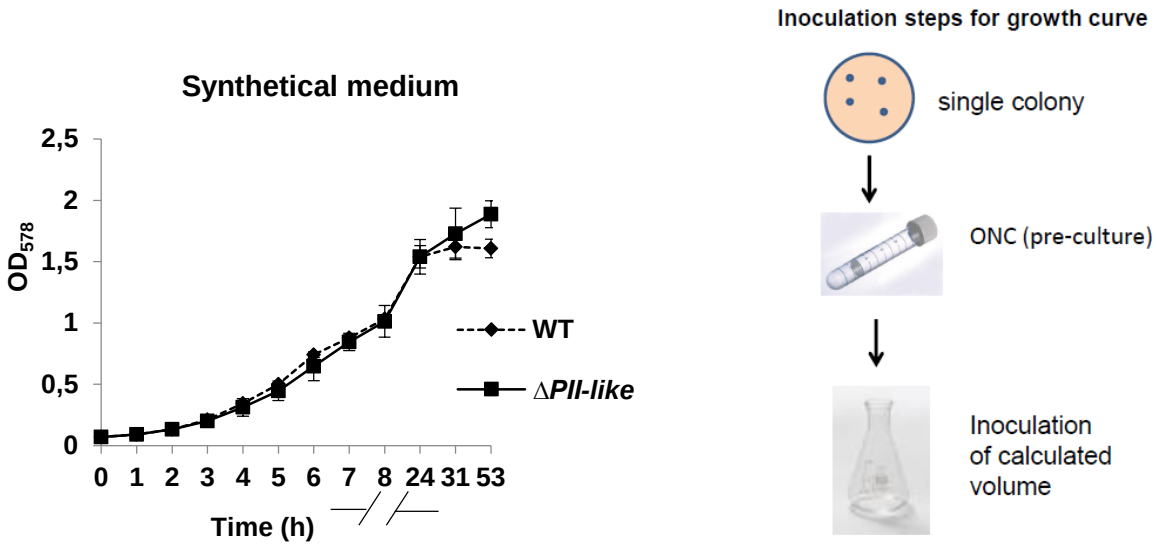


Figure 21. Growth in synthetical medium with first inoculation step

Single colonies of WT and Δ*PII-like* strains were inoculated into a synthetical medium for overnight culture and this overnight culture was used to start growth analysis.

Growth studies in synthetical medium were performed under aerobic conditions and shaking by 150 rpm at 37 °C. Overnight cultures, were inoculated with a single colony grown on agar plates containing rich media into synthetical medium and this overnight culture was used as first inoculant to investigate growth properties. Growth was very slow and no difference could be observed between WT and Δ*PII-like* mutant strains (Fig. 21.).

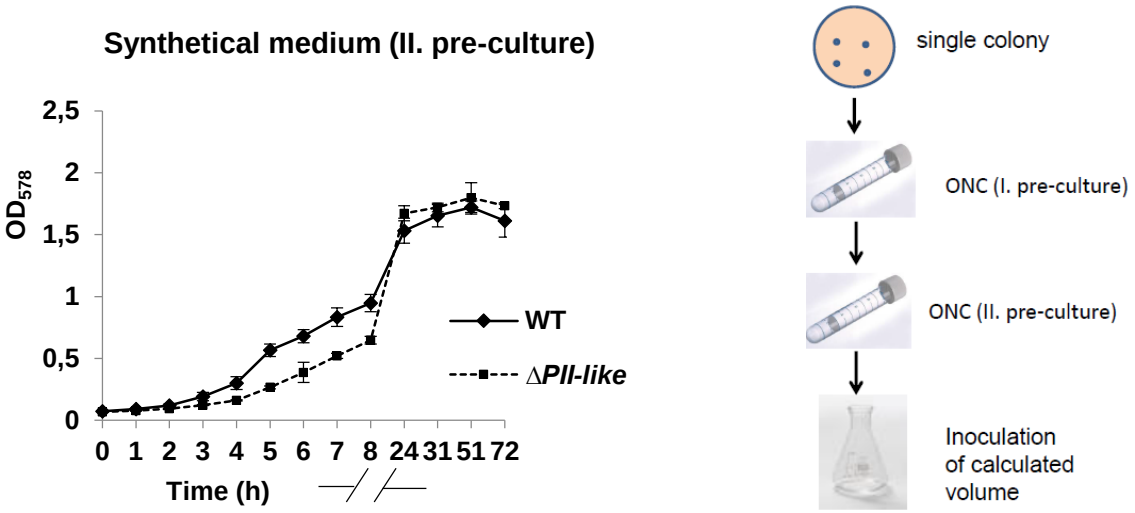


Figure 22. Growth in synthetical medium with second inoculation step

The first overnight culture with a single colony was inoculated for the second overnight culture and used for growth analysis in synthetically medium.

The first liquid culture (I. pre-culture) was used as inoculant and freshly inoculated into new synthetical medium (II. pre-culture) to start growth studies. Interestingly, Δ*PII-like* mutants showed a growth delay again, as observed in complex media during the exponentially phase of growth. In addition, no significant difference was observed when the cells reached the stationary phase (Fig. 21.).

D.2.1.3 Aerobically/anaerobically growth in the presence or absence of $NaNO_3$ (25 mM) and accumulation of nitrite in medium

To characterize the nitrate-reducing system in *S. aureus*, the nitrate reduction was determined during growth. Growth analysis were performed in TSB medium. WT and Δ*PII-like* cells were grown in the presence of oxygen with/without $NaNO_3$ (25 mM) (Fig. 23. a-b) and showed no significant growth differences. Interestingly, growth in the presence of $NaNO_3$ was lower at the end of exponential phase compared to growth without $NaNO_3$. However, in the absence of oxygen, many bacteria preferentially use nitrate as a terminal electron acceptor (Neubauer & Götz, 1996). Therefore, same growth analysis was performed under the anoxic conditions again. Similar growth delays were observed in the presence of $NaNO_3$ during the anaerobic growth.

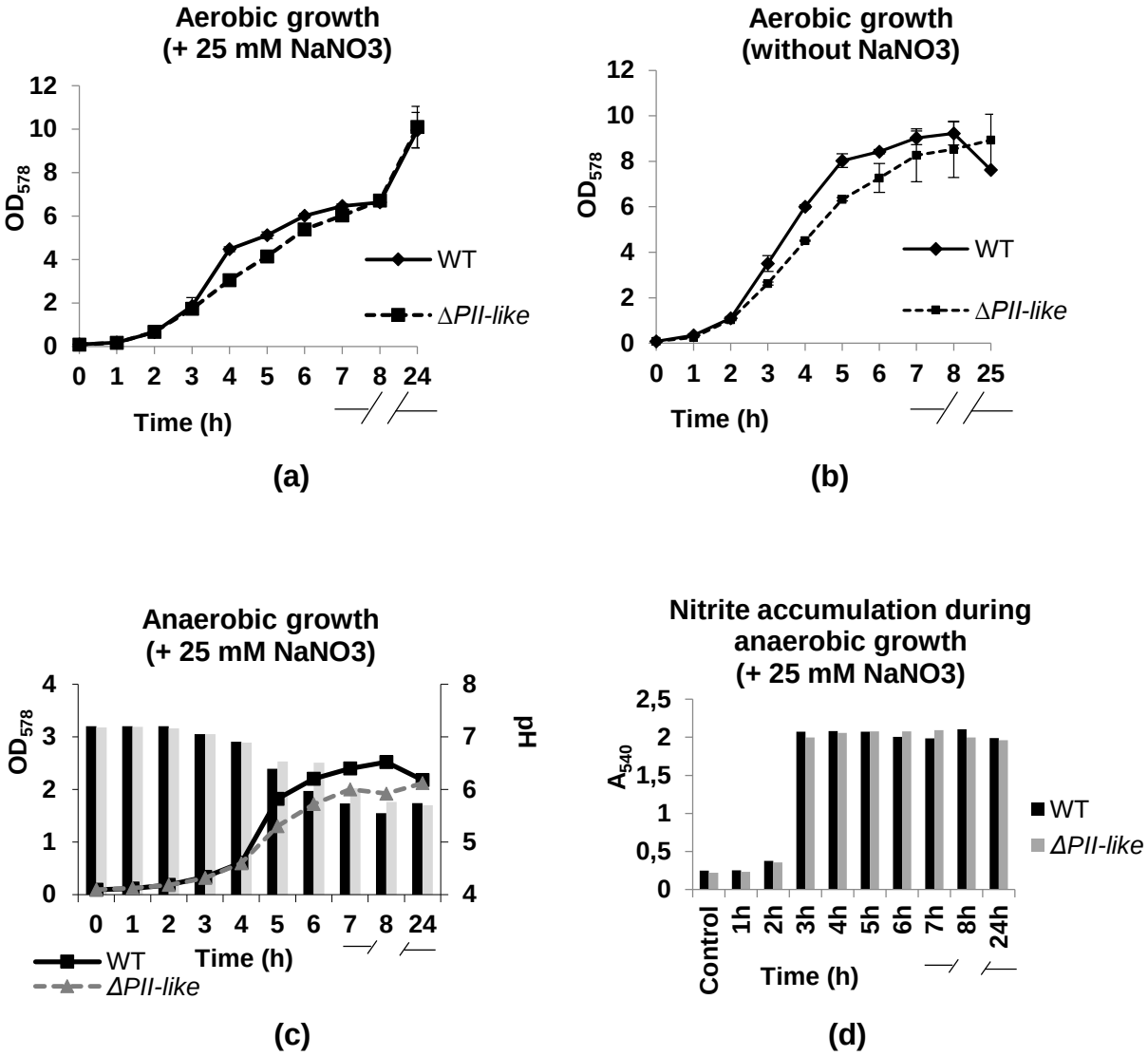


Figure 23. Growth under anaerobically/aerobically conditions (with-without $NaNO_3$) and accumulation of nitrite in medium

WT and Δ*PII-like* mutant cells were grown differentially during the exponentially phase in the presence or absence of $NaNO_3$.

Additionally, pH values were measured during the growth and no pH change was observed between WT and Δ*PII-like* mutant cultures (Fig. 23. c). Moreover, we observed that cells are reducing nitrate to nitrite and then accumulates nitrite in the medium. Unfortunately, there was no difference in accumulation of nitrite in medium between the WT and its Δ*PII-like* mutant strain (Fig. 23. d).

D.2.1.4 Investigation of growth after dilutions

Thus, analysis of growth with the normal amount of cells did not showed any significant difference. To study this defect or delayed growth in more detail, we investigated the effect of low cell numbers in the course of long adaptation times.

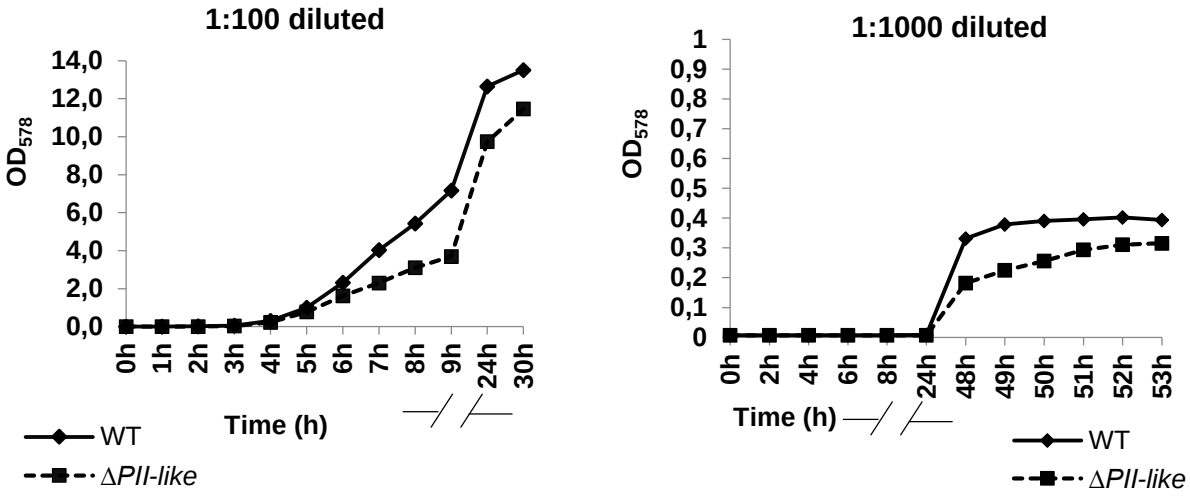


Figure 24. Growth with diluted cultures (1:100, 1:1000) to find more phenotypic effect

WT and Δ*PII-like* mutant cells showed similar growth difference compared of growth with undiluted cultures.

Growth analysis with serially diluted overnight cultures (dilutions: 1:100 and 1:1000) were carried out. Unfortunately, we could not observe similar growth delays (Fig. 24.). The cell number did not show any effect on the growth characteristics of both strains.

D.2.2 Characterization of nitrate reduction

D.2.2.1 Nitrate reductase activity

The influence of *PII-like* deletion on the dissimilation of nitrate reduction in staphylococci was investigated by testing the nitrate reductase activity.

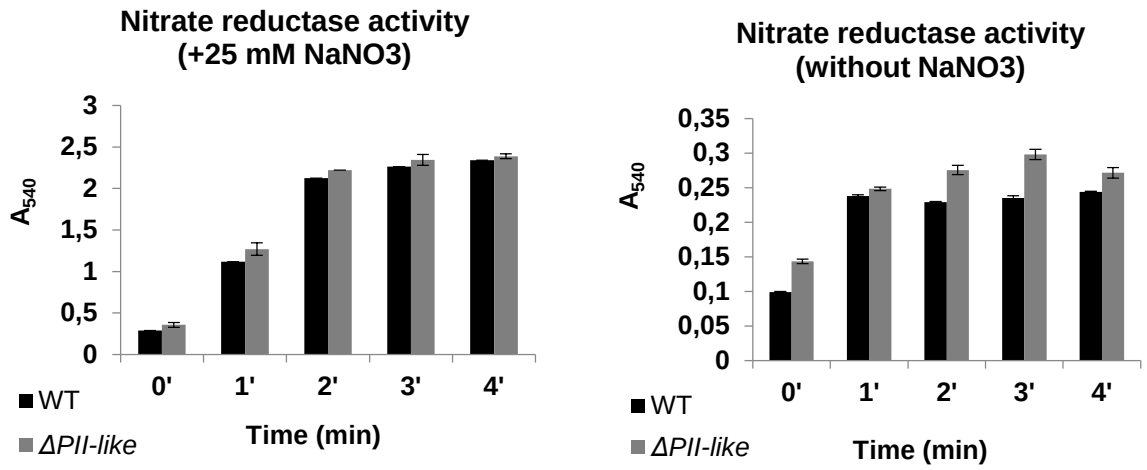


Figure 25. Nitrate reductase activities under the anaerobic conditions (with/without $NaNO_3$)

A similar nitrate reductase behavior was observed between WT and Δ*PII-like* strains. Cells were cultivated aerobically or anaerobically in the presence or absence of $NaNO_3$. After the cells were harvested, they were washed twice in potassium phosphate buffer (100 mM, pH 7.2) and resuspended in the same buffer.

Therefore, cultures were grown in TSB medium under anaerobic conditions in the presence or absence of $NaNO_3$. Cells were harvested in the mid-exponential phase and used for a nitrate reductase assay according to the protocol of Neubauer and Götz, 1996 (Neubauer & Götz, 1996). The activity of nitrate reductase with anaerobically grown cells in the presence and absence of $NaNO_3$ did not show any difference between WT and Δ*PII-like* mutant cells (Fig. 25.).

D.3 Analysis of biofilm formation

For the investigation of biofilm formation, TSB was used as medium and analysis was performed in 96-well polystyrene microtiter plates (Schlag *et al.*, 2007). Interestingly, Δ*PII-like* mutants exhibited an increased biofilm formation compared to the WT strain. Additionally, a higher biofilm formation in first 8h was observed compared to 24h (Fig. 26.).

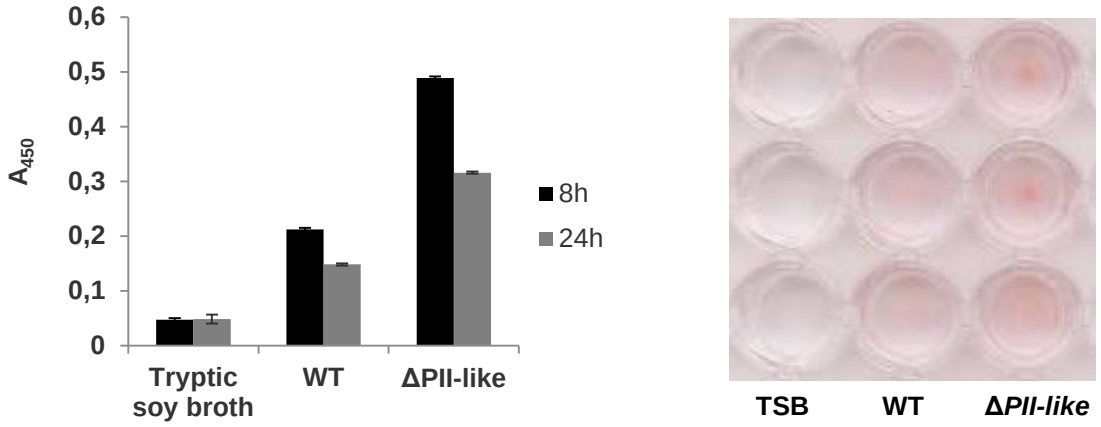


Figure 26. Biofilm formation of wild type and Δ*PII-like* mutant strains

The Δ*PII-like* mutant showed an increased biofilm formation compared to the WT strain. Cells were grown in TSB at 37°C for 24 h. Biofilm cell layers were visualized by safranin staining and measured at 450 nm. Microtiter plate presented are representative of at least three independent sets of experiments.

D.4 *PII-like* protein purification

D.4.1 Construction of overexpression vector

A synthetic *S. aureus PII-like* gene with optimized codon usage for *E. coli* expression was synthesized by Geneart/Life Technologies, Germany. The DNA sequence originates from the amino acid sequence of the potential *PII-like* protein encoded within the *S. aureus* genome. A Strep tag II sequence was fused to the *PII-like* sequence at the C-terminus. The synthetic gene *PII-like* in pMA-T vector was digested with *Eco*31I (*Bsa*I) and cloned into the anhydrotetracycline (ATc) inducible pASK-IBA3 vector. The final over-expression vector pASK-IBA3_*PII-like*-Strep II (Fig. 27.) was transformed into *E. coli* BL21 (DE3) cells.

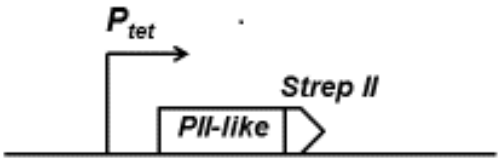


Figure 27. Overexpression vector pASK-IBA3_*PII-like*-Strep tag

PII-like is C-terminally fused with a Strep tag II on the vector pASK-IBA3 and was inserted through *Eco*31I (*Bsa*I) site.

D.4.2 Purification of *PII-like* and SDS-PAGE analysis

Overexpression of recombinant *PII-like* protein was carried out in *E. coli* BL21 (DE3). Cells were induced with 200 µg/L ATc at OD_{578} 0.6-0.8 and grown overnight at 37°C. Recombinant proteins were purified on a Strep-tactin superflow column according to manufacturer's protocol (Fig. 28.).

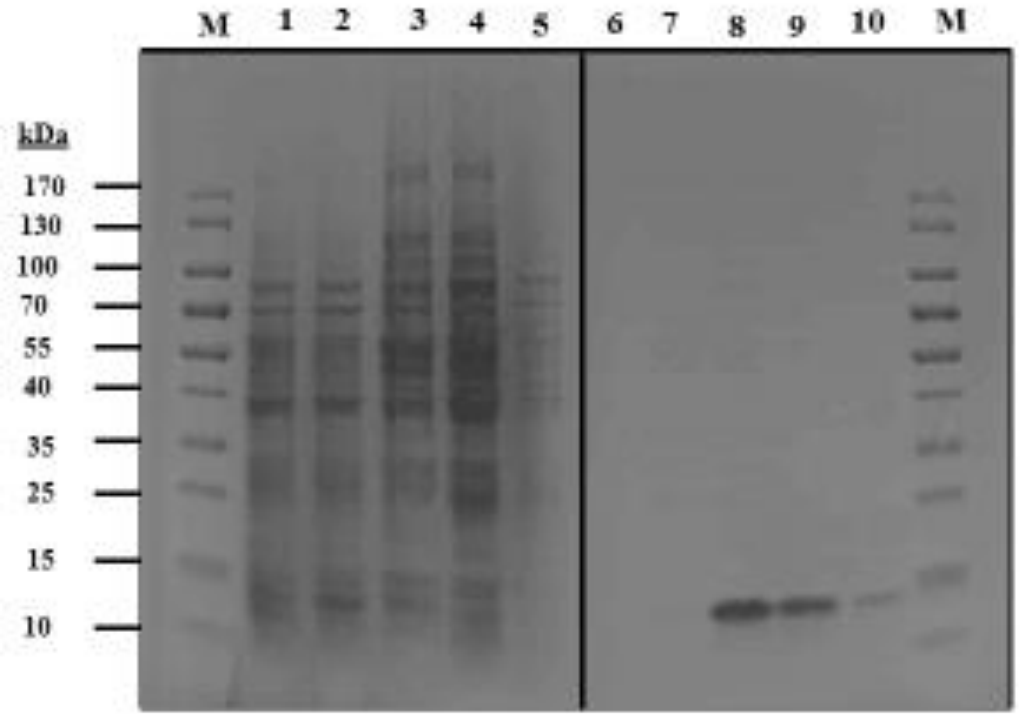


Figure 28. Overexpression and purification of Strep II tagged *PII-like*

M, Molecular weight standard; Lane 1) uninduced crude extract, 2) induced crude extract-I, 3) induced crude extract-II, 4) Flow through-I, 5) Flow through-II, 6) Elution-I, 7) Elution-II, 8) Elution-III, 9) Elution-IV, 10) Elution-V

D.5 Binding studies using Isothermal titration calorimetry

D.5.1 Interaction of *PII-like* with c-di-AMP and cAMP

Purified *PII-like* proteins were dialyzed overnight in Buffer-T containing 20 mM Tris-HCl, 150 mM NaCl (pH 7.5). c-di-AMP solution was prepared by dilution of a 1 mM stock solution (in water) with Buffer-T. 130 µM of c-di-AMP solution was injected into a 40 µM of *PII-like* solution at 24 °C. Data were fitted using the "*two-binding-site model*" of the MicroCal version of ORIGIN. The heat of binding (Δ*H*), the stoichiometry (*n*), the

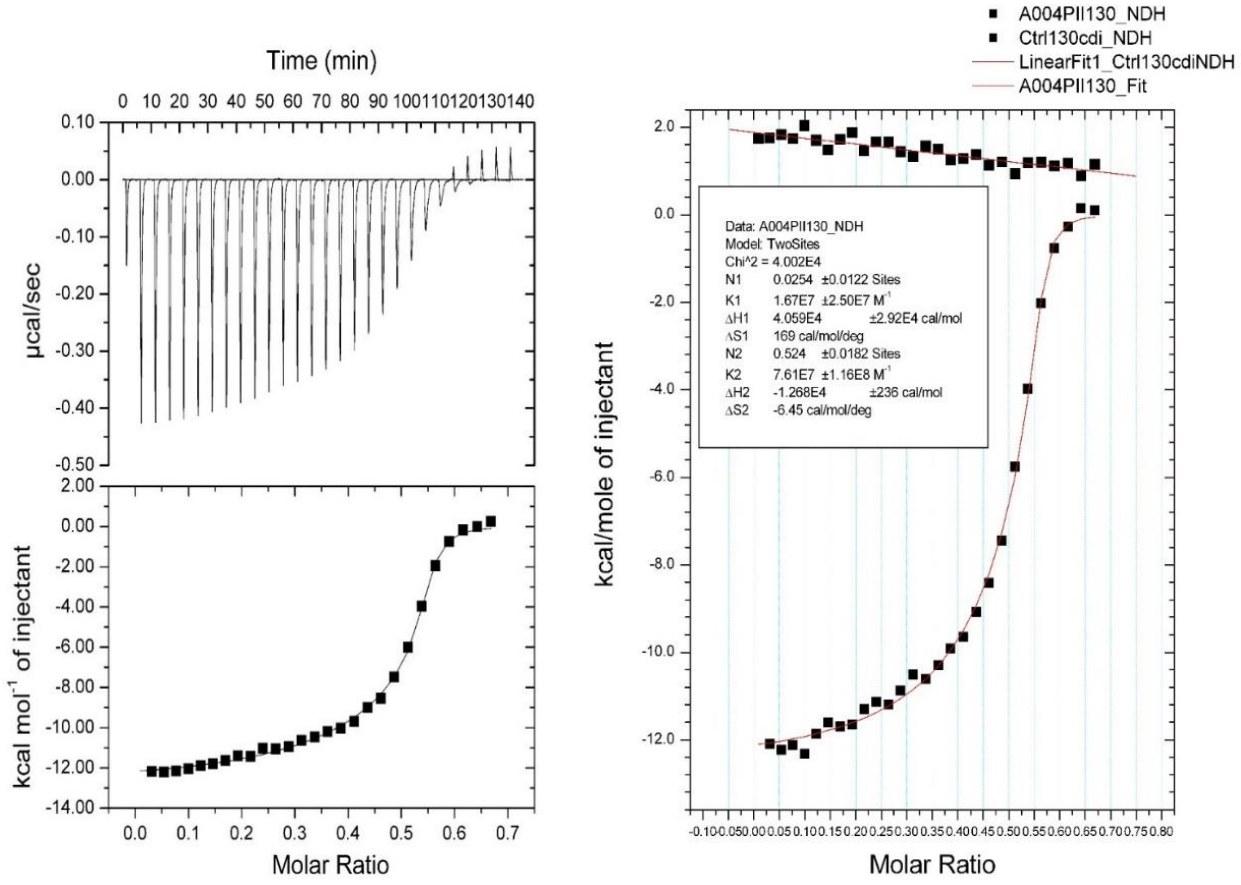
association constant (K_a) and the dissociation constant (K_d) were calculated from the plot of the heat.

Table D.1 The values of calculated c-di-AMP association/dissociation constants for the *PII-like* binding sites 1-2 and ± SEM are shown.

Binding site	K_a1 [µM]	K_d1 [µM]	SEM
1	16,7	0,06	± 25

Binding site	K_a2 [µM]	K_d2 [µM]	SEM
2	76,1	0,013	± 116

A strong binding of c-di-AMP was observed under optimal binding conditions, which could be perfectly fitted with a *two sequential binding* sites model.



(a) **(b)**

Figure 29. Binding of *PII-like* with c-di-AMP

(a) These panels shows the raw data as a heat effect during the titration of 40 µM *PII-like* solution (trimer concentration) into c-di-AMP solution. **(b)** The panel shows binding isotherm and the best fit curve according to the model of *two sequential binding* sites after subtraction of buffer effect.

Data analysis resolved one low-affinity binding site (association constant for the first site: K_a1= 16,7 ± 25 µM and the dissociation constant K_d1= 0,07 µM) and one high-affinity site (site 2) (association constant for the first site: K_a2= 76,1 ± 116 µM and the dissociation constant K_d2= 0,013 µM) (Fig. 29. a-b) (Table D.1).

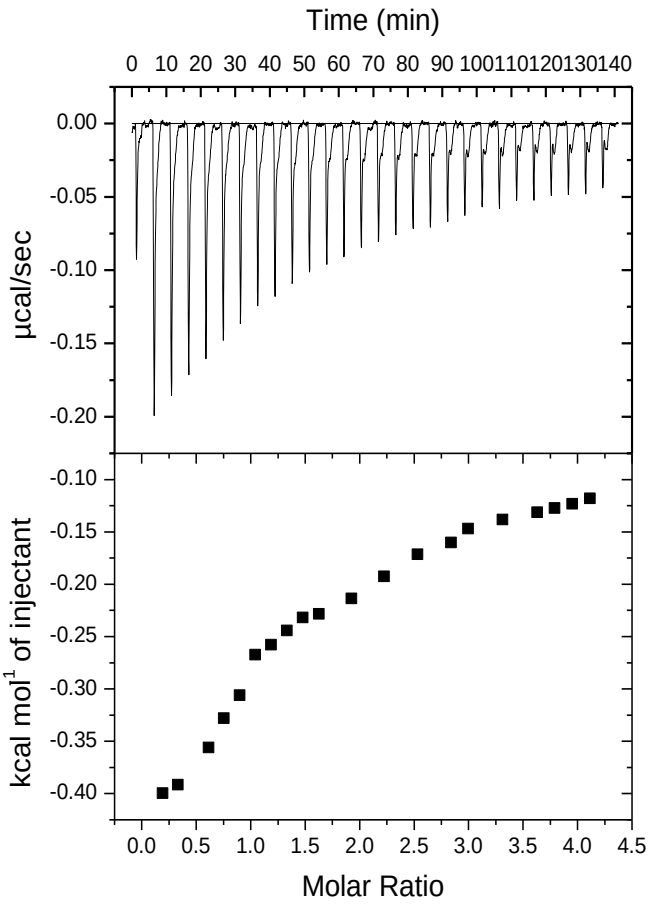


Figure 30. Binding of *PII-like* with cAMP

The binding of another nucleotide, cAMP, to *PII-like* was determined again using ITC. We observed a very weak interaction between cAMP compared to c-di-AMP nucleotide. The panel shows the raw data as a heat effect during the titration of 100 µM *PII-like* solution with 2 mM cAMP. The upper panel shows the peaks of the corrected heat rate produced by the successive injection of cAMP to *PII-like* solution over time (Fig. 30.).

D.6 Verification of c-di-AMP and cAMP binding using Biacore surface plasmon resonance

S. aureus PII-like was coupled to a CM-5 chip *via* amine coupling chemistry. To increase the sensitivity of the nucleotide binding, we saturated the chip surface with *PII-like*. Kinetic

data were recorded for injections of c-di-AMP and cAMP in running buffer (20 mM Tris-HCl, 150 mM NaCl, % 0.05 Tween, pH 7.5). Data from an empty flow cell (FC1) were subtracted as reference to correct unspecific bindings of the analytes to the chip surface. Data were analyzed using the Biacore X100 Evaluation software.

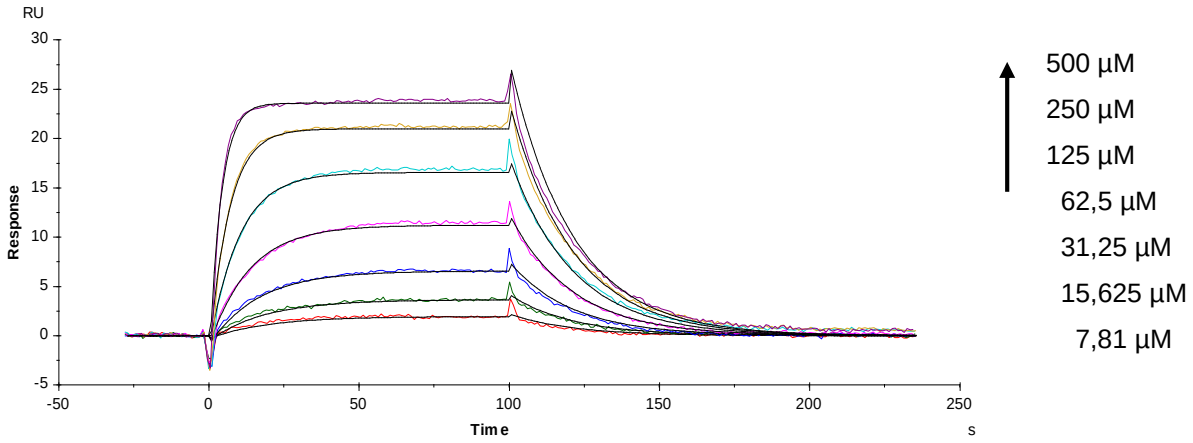


Figure 31. Injections of different concentrations of c-di-AMP (colored curves) molecules over the immobilized *PII-like* chip-surface (FC2-1)

The sensorgrams displays binding curves over the entire course of an interaction and reveals association and dissociation rates of the interaction

Depending the concentrations of c-di-AMP, different response units (RU) were obtained. An expected value for calculated RU_{max} after interaction between *PII-like* and c-di-AMP is around 56 RU. In contrast, our Biacore X100 experiments, showed RU_{max} values of approximately 28 RU at a concentration of 500 µM after interaction of *PII-like* and c-di-AMP (Fig. 31.). *PII-like* displays robust binding to c-di-AMP with K_D= 92,1 µM, an association rate of k_a= 4,45 x 10^2 $M^{-1}s^{-1}$ and a slow dissociation rate of k_d= 0.04 s^{-1} (Table D.2).

Table D.2 Kinetic data of interaction for *PII-like* / c-di-AMP

k_a (1/M s) Association rate constant	**k_d (1/s)** diassociation rate constant	**K_D (M)** equilibrium dissociation constant (affinity)	**Rmax (RU)** Analyte binding capacity of the surface
445,0872061 (4,45 x 10^2)	0,040973	9,21E-05 (92 nM)	27,9173854

In addition, we investigated binding kinetics of cAMP to *PII-like* with different concentrations. Again, depending on the concentrations of cAMP, we observed different response units (Fig. 32.).

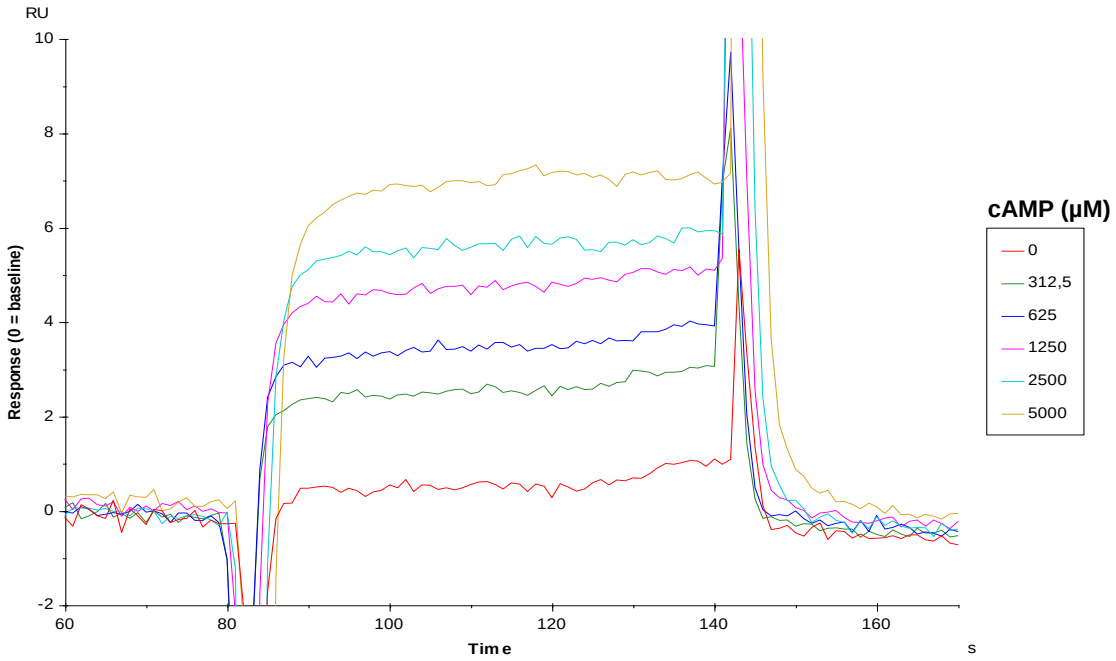


Figure 32. Injections of different concentrations of cAMP (colored curves) molecules over the immobilized *PII-like* chip-surface (FC2-1)

Expected RU_{max} for the interaction between *PII-like* and cAMP has to be 28 RU. We could calculate a value of approximately 6,8 RU at a concentration of 5 mM cAMP. *PII-like* displays a weak binding to cAMP compared to c-di-AMP, with a very strong affinity (K_D= 1472 nM) (Table D.3).

Table D.3 Kinetic data of interaction for *PII-like* / cAMP

K_D (M) equilibrium dissociation constant (affinity)	**Rmax (RU)** Analyte binding capacity of the surface
0,001472 (1472 nM)	6,807018

D.6.1 Investigation of interaction with other nucleotides

PII/PII-like interactions are modulated by binding of the effector molecules such as ATP, ADP and 2-OG. Therefore, we analyzed the ATP-, ADP-, AMP-, GTP- and TTP- binding properties of *PII-like* using Biacore surface plasmon resonance. The injections of ATP, ADP, AMP, GTP and TTP were performed under the same conditions. Interestingly, we obtained an unclear binding signal only with ATP sample (Fig. 33.).

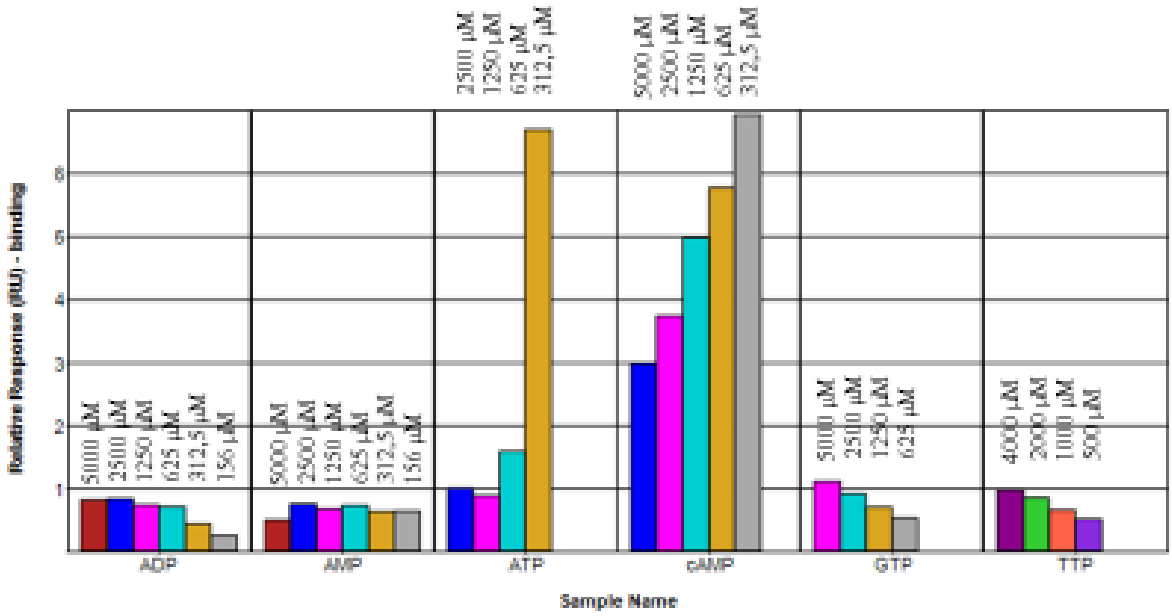


Figure 33. Analysis of ATP-, ADP-, AMP-, GTP- and TTP- binding properties

E. DISCUSSION

E.1 Effects of *PII-like* absence on growth characteristic

For bacterial *PII-like* proteins, which have been reported to play important roles as sensor and signal transducers in nitrogen control, several receptor proteins involved in nitrogen metabolism have been described. The activity of those is often modulated by direct protein interaction with the respective *PII-like* proteins (Arcondeguy *et al.*, 2001). Well studied examples are *E. coli* GlnB, which transduces the nitrogen signal towards the sensor kinase NtrB (Jiang *et al.*, 1998) and towards the glutamine synthetase (Reitzer, 2003), *E. coli* GlnK, which negatively regulates the activity of ammonium transporter AmtB by protein protein interaction (Coutts *et al.*, 2002) and the *Synechococcus elongatus* PII protein, which forms tight complexes with the N-acetylglutamate kinase under nitrogen excess conditions and thereby enhances its enzyme activity (Heinrich *et al.*, 2006). In contrast to the bacterial *PII-like* proteins, the knowledge about archaeal *PII-like* proteins concerning their regulatory functions and even more the understanding of the respective regulatory mechanisms is rather limited.

As demonstrated by Atkinson and Ninfa (1998), GlnK is required to regulate expression of Ntr genes in cells that lack PII. In the absence of this regulation by GlnK, cells have a severe growth defect on defined medium that is correlated with overexpression of the Ntr regulon (Atkinson & Ninfa, 1998). Furthermore, PII and *PII-like* exhibit different structural and functional features. However, in this work we did not investigate Ntr expression but the deletion of *PII-like* in *S. aureus* created a viable mutant showing a growth defect which was expected, because of the absence of nitrogen regulation proteins in mutant cells. Blauwkamp and Ninfa (2002) reported that cells lacking either PII or GlnK do not show any clear growth defect, but cells lacking both PII and GlnK demonstrate a very dramatic growth defect on minimal medium, which is complemented by supplementation of the medium with a complex mixture of amino acids (Blauwkamp & Ninfa, 2002).

In order to address the question, how *PII-like* is involved in nitrogen regulation, we investigated physiological properties of the *PII-like* mutant *S. aureus* strain and firstly the growth characteristics were compared in rich and synthetic media. When cells were growing in rich or synthetic medium, no significant phenotype of the mutant strain was detectable compared to WT. However, a partial growth defect or a delayed growth of the mutant strain was observed at the exponential phase. This finding indicates that the

PII-like protein is required for maximal growth during the exponential phase, but it is not essential for viability. In addition, dilution series were performed to observe a possible effect of dilutions of the cell number on growth differences. Interestingly, our ∆*PII-like* mutant skipped almost completely the *lag* phase, but started to grow slowly in the beginning of the *log* phase. A possible explanation for this growth behavior that whatever protein it is that takes over the function of *PII-like* during the exponential phase. When the cells reached the stationary phase, the OD of both strains was the same. To have a closer look, the cultures were inoculated with dilution series during the growth which revealed that the decreased OD might be due to dying mutant cells than a slower growth.

Certain bacteria, such as *E. coli* and *Salmonella typhimurium*, do not assimilate nitrogen by reducing nitrate during aerobic growth but grow anaerobically with nitrate as the terminal electron acceptor. Neubauer and Götz (1996) demonstrated that under anoxic conditions, cells of *S. carnosus* rapidly reduce nitrate to nitrite, which accumulates in the external medium (Neubauer & Götz, 1996). In our experiments, nitrate reduction was determined in the course of growth in the absence of $NaNO_3$ and under aerobic/anaerobic conditions. Accumulated nitrite concentration in the external medium was measured and no significant change was observed between WT and ∆*PII-like* mutant. Additionally, the effect of $NaNO_3$ was tested on aerobic/anaerobic grown cells and no significance was demonstrated, as well. These results indicated that the lack of *PII-like* has not any influence on nitrate reduction and growth characteristics. In addition, these results were confirmed by nitrate reductase assay.

E.2 Influence of *PII-like* absence on nitrate reductase activity

Many bacteria are able to use nitrate as a source of nitrogen or as a terminal electron acceptor under anoxic conditions (nitrate respiration). In the assimilatory process, which may occur under aerobic or anaerobic conditions, nitrate is finally reduced to ammonia (NH_3) and subsequently incorporated into biomass (Neubauer & Götz, 1996). Ohashi *et al.* (2011) were reported that PII protein inhibits the ABC-type nitrate transporter and also nitrate reductase in some strains, by unknown mechanisms at low cellular 2-OG levels (Ohashi *et al.*, 2011). Also Zhang et *al.* (2007), showed that PII mutant of *Anabaena* sp. PCC 7120 grows poorly in nitrate-containing medium and excretes ammonium into the medium. Finally they demonstrated an increased activity of nitrate reductase and reduced activity of glutamine synthetase in PII mutants of *Anabaena* sp. PCC 7120 (Zhang *et al.*,

2007). The influence of the absence of *PII-like* on the dissimilation of nitrate reduction in *S. aureus* was investigated and nitrate reductase activity of Δ*PII-like* mutant cells were analyzed. The effect of *PII-like* absence on anaerobically grown cells in the presence of $NaNO_3$ did not shown any difference between the WT and Δ*PII-like* mutant cells. In almost all Cyanobacteria, the global nitrogen control is consumed by a transcription factor, NtcA. NtcA mediates nitrogen control activating transcription of nitrate assimilation genes at high C to N ratio, which is signaled by the 2-OG levels (Herrero *et al.*, 2001, Flores *et al.*, 2005). The PII protein is a 2-OG sensor that required for the expression of NtcA-dependent genes under nitrogen deficiency (Aldehni & Forchhammer, 2006, Forchhammer, 2004). Here, PII proteins are acting nitrate reductase in various steps indirectly under different nitrogen/carbon source conditions. In addition, our results indicates that nitrate reductase in *S. aureus* is not regulated directly by *PII-like* and can be explained with other regulation processes.

E.3 Binding of *PII-like* with c-di-AMP and cAMP

The present study aimed to identify a *PII-like* protein in *S. aureus* and to characterize functional similarities between other well-studied PII signal transduction protein. The *PII-like* protein was identified as a receptor protein for the seconder messenger molecule c-di-AMP. Several *PII-like* proteins have been identified and solved in complex with the second messenger nucleotide c-di-AMP (Müller *et al.*, 2015, Campeotto *et al.*, 2014, Gundlach *et al.*, 2014). Campeotto *et al*. (2014), presented a target protein for c-di-AMP, which is called the *S. aureus PII-like* signal transduction protein PstA. They were not able to establish a classical consensus sequence for PstA protein and suggested a prototype of a new protein family with clearly distinct features from classical PII proteins. Additionally, they proposed that comparative analysis of the apo and complex structures allows to present a model how PstA functions as a signal transduction protein (Campeotto *et al.*, 2014). Furthermore, Gundlach *et al*. (2014) identified the *PII-like* signal transduction protein DarA in *B. subtilis* as a target of the c-di-AMP. Crystallization studies demonstrates that DarA is indeed a *PII-like* protein forming the canonical PII homotrimer. They found significant differences for the B and T loops and importantly observed that these loops are swapped with respect to their size (Gundlach *et al.*, 2014).

In order to analyze the binding of various nucleotides to *PII-like* we performed ITC and Biacore surface plasmon resonance experiments. The resulted data clearly confirm the

binding of c-di-AMP to *PII-like*. We obtained data that suggest a 1:1 binding stoichiometry and interaction with c-di-AMP was in exergonic character. The binding and 1:1 stoichiometry can be clearly displayed as proof-of principle in quantitative data analysis. The experiments shown in Fig. 29 and Fig. 30. indicated that the *PII-like* protein responded to the secondary messenger molecules c-di-AMP and cAMP quite differently. The interaction measurements were performed with different ligand-protein concentrations to determine the conditions under which the best fitting to the binding model could be achieved. Subsequently, the experiments were repeated to confirm these results. As shown in Fig. 29, *PII-like* protein exhibits high affinity toward c-di-AMP. Optimal fitting of the raw data was obtained using a *two sequential binding* sites model (Fig. 29a, lower part). This analysis revealed a low association constant (K_a) for the first binding site (K_a1= 16,7 ± 25 µM) and increased K_a for binding sites 2 (K_a2= 76,1 ± 116 µM). Interestingly, the K_a of *PII-like* towards c-di-AMP was increased fourfold at the second binding site, compared to first site. On the other hand, the dissociation constant of first binding site (K_d1= 0,06 µM) was showed a high-affinity compared to the dissociation constant of second binding site (K_d2= 0,013 µM). A direct comparison of the ITC data under identical conditions suggests *PII-like* to bind c-di-AMP with higher affinity and to cAMP with lower affinity. This results indicates that the activities of c-di-AMP and cAMP molecules are affecting by *PII-like* in *S. aureus* cells with unknown mechanisms in cellular context. We have further investigated various nucleotides, which could potentially act as *PII-like* effectors: ATP, ADP, AMP, GTP and TTP. Whereas we could not detect any clear interaction between ATP, ADP, AMP, GTP and TTP nucleotides.

In order to quantify the affinity and kinetic properties of nucleotides which binding to *PII-like*, we performed Biacore surface plasmon resonance experiments. Amino-reactive coupling chemistry was used to covalently immobilize *PII-like* to the chip surface and increasing concentrations of nucleotides in Buffer-T were injected as analyte (Fig. 31-32). *PII-like* exhibits strong binding to the c-di-AMP with an affinity K_D = 92,1 µM, an association rate of ka = 4,45 x 10^2 $M^{-1}s^{-1}$ and a slow dissociation rate of kd = 0.04 s^{-1}. The calculated RU_{max} for the interaction of *PII-like* and c-di-AMP should be 56 RU. But we observed a 28 RU with a 50% decrease of binding capacity. Thus, the decrease of RU in c-di-AMP-binding sensorgrams indicates that c-di-AMP binding is much slowly than expected. We also performed injections with ATP, ADP, AMP, GTP, TTP and cAMP (Fig. 33). Only the cAMP was showed a significant binding to *PII-like*, with a weak response

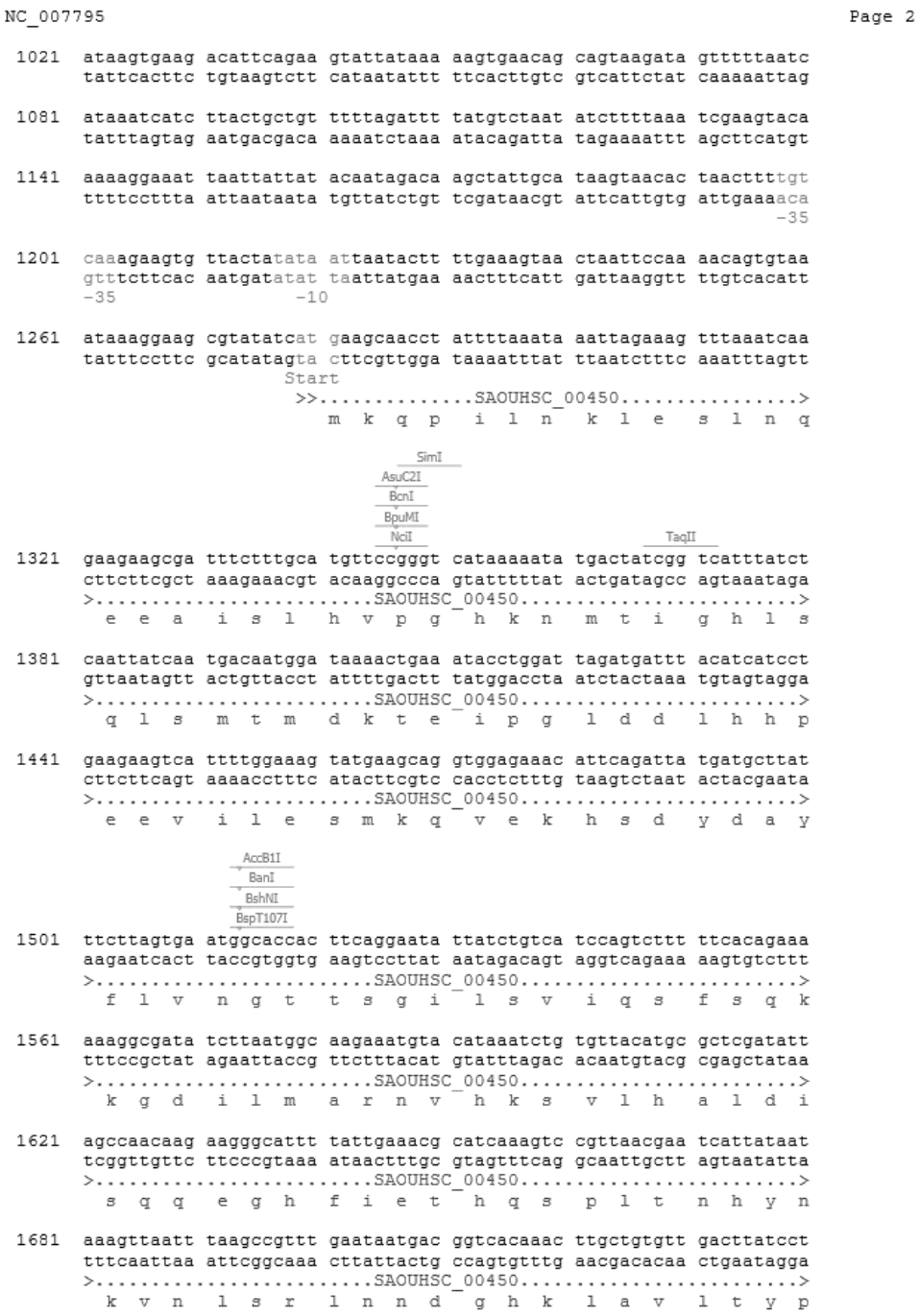
RU_{max}: 6,8 but with a much higher affinity (K_D = 1472 nM). Additionally, no binding was detected towards ADP, AMP, TTP and GTP under the used conditions. Interestingly, any interaction between ATP (312,5 µM) was observed with unexplainable data and this interaction could not be fitted with any binding model. Also, this data could not be confirmed with the results of ITC.

E.4 Does *PII-like* protein affects biofilm formation?

Biofilm formation has been determined as one of the most important survival strategies in bacteria and this is used by the cell to protect against predators and antibiotics (Wang *et al.*, 2005). The genetic and molecular basis of biofilm formation is regulated in a multifactorial process. Initially, attachment can be mediated by various cell surface-associated factors such as major autolysins, teichoic acids or polysaccharide intercellular adhesins (PIA), the product of the *ica*ADBC gene cluster (Schlag *et al.*, 2007, Götz, 2002). Here we report an exhibited biofilm formation in *PII-like* deficient cells. Until today no comparative studies were published, where a direct link between PII/*PII-like* protein and biofilm formation could be shown. Ardin *et al.* (2014) characterized the ammonium transporter protein and its operon in the Gram-positive bacterium *Streptococcus mutans* (*S. mutans*). They reported that the SMU.1658 gene corresponding to *nrg*A in *S. mutans* is homologous to the ammonium transporter gene in *Bacillus subtilis* and SMU.1657, located upstream of the *nrg*A gene and predicted to be *gln*B, is a member of the PII protein family. Their findings suggest that the *nrg*A gene in *S. mutans* is essential for export of molecules and biofilm formation, while the *gln*B gene, which might be related to the function of the nitrogen regulatory protein PII, may activate expression of the *nrg*A gene (Ardin *et al.*, 2014). We know that cAMP regulates not only catabolite regulation, but also biofilm formation, quorum sensing, and nitrogen regulation (Shimizu, 2013). An increase of biofilm formation in ∆*PII-like* is not explainable with a deficiency of *PII-like*. Therefore, this multifactorial process and link with nitrogen regulation still need to be investigated in *S. aureus*.

F. SEQUENCES

F.1 Gene sequence of SAOUHSC_00452 encoding for a putative *PII-like* of *S. aureus*



```
1741  aactattacg gtgaaacatt taatgtagaa gaggttatca aatctttgca ccaattaaat
      ttgataatgc cactttgtaa attacatctt ctccaatagt ttagaaacgt ggttaattta
      >.......................SAOUHSC_00450.......................>
        n  y  y   g  e  t   f  n  v  e   e  v  i   k  s  l   h  q  l  n

                                      BsePI
                                     BssHII
                                      PauI
1801  attcctgtac tcattgacga agcacacggc gcgcactttg gattgcaagg atttccagat
      taaggacatg agtaactgct tcgtgtgccg cgcgtgaaac ctaacgttcc taaaggtcta
      >.......................SAOUHSC_00450.......................>
        i  p  v   l  i  d   e  a  h  g   a  h  f   g  l  q   g  f  p  d

1861  tctacattaa attatcaagc tgactatgtt gttcaatctt ttcataaaac gttaccagct
      agatgtaatt taatagttcg actgatacaa caagttagaa aagtattttg caatggtcga
      >.......................SAOUHSC_00450.......................>
        s  t  l   n  y  q   a  d  y  v   v  q  s   f  h  k   t  l  p  a

1921  ttaacgatgg gctcggtact ttatattcat aaaaatgcac cttatagaga aactattata
      aattgctacc cgagccatga aatataagta tttttacgtg gaatatctct ttgataatat
      >.......................SAOUHSC_00450.......................>
        l  t  m   g  s  v   l  y  i  h   k  n  a   p  y  r   e  t  i  i

                                                                MlyI
                                                                PleI
                                                                PpsI
                                                                SchI
1981  gaatatctaa gctacttcca aacatctagt ccttcgtatt tgattatggc tagtttagag
      cttatagatt cgatgaaggt ttgtagatca ggaagcataa actaataccg atcaaatctc
      >.......................SAOUHSC_00450.......................>
        e  y  l   s  y  f   q  t  s  s   p  s  y   l  i  m   a  s  l  e

            NmeAIII
2041  tcagctgccg agttctataa aacatatgat agtaccgtgt tttttgataa gagagcgcaa
      agtcgacggc tcaagatatt ttgtatacta tcatggcaca aaaaactatt ctctcgcgtt
      >.......................SAOUHSC_00450.......................>
        s  a  a   e  f  y   k  t  y  d   s  t  v   f  f  d   k  r  a  q

2101  ttaatcgaat gtttggagaa gaagggtttt gaaatgcttc aagttgatga tccgttgaag
      aattagctta caaacctctt cttcccaaaa ctttacgaag ttcaactact aggcaacttc
      >.......................SAOUHSC_00450.......................>
        l  i  e   c  l  e   k  k  g  f   e  m  l   q  v  d   d  p  l  k

2161  ttgctgataa aatatgaagg ttttacaggt catgatattc aaaattggtt tatgaatgca
      aacgactatt ttatacttcc aaaatgtcca gtactataag ttttaaccaa atacttacgt
      >.......................SAOUHSC_00450.......................>
        l  l  i   k  y  e   g  f  t  g   h  d  i   q  n  w   f  m  n  a

2221  catatctatt tagaattagc ggacgactat caagcattag cgatattgcc gttatggcat
      gtatagataa atcttaatcg cctgctgata gttcgtaatc gctataacgg caataccgta
      >.......................SAOUHSC_00450.......................>
        h  i  y   l  e  l   a  d  d  y   q  a  l   a  i  l   p  l  w  h

2281  catgatgata cgtatttatt tgattcgctt ttacgtaaaa ttgaagatat gattttaccg
      gtactactat gcataaataa actaagcgaa aatgcatttt aacttctata ctaaaatggc
      >.......................SAOUHSC_00450.......................>
        h  d  d   t  y  l   f  d  s  l   l  r  k   i  e  d   m  i  l  p

2341  aaaaaatcag tttctaaagt taaacaaaca caacttttaa caactgaagg taactataaa
      ttttttagtc aaagatttca atttgtttgt gttgaaaatt gttgacttcc attgatattt
      >.......................SAOUHSC_00450.......................>
        k  k  s   v  s  k   v  k  q  t   q  l  l   t  t  e   g  n  y  k
```

```
2401  ccaaaacgct ttgaatatgt tacttggtgt gatttgaaaa aggcaaaagg taaagttctg
      ggttttgcga aacttataca atgaaccaca ctaaactttt tccgttttcc atttcaagac
      >.........................SAOUHSC_00450.........................>
        p  k  r   f  e  y   v  t  w  c   d  l  k   k  a  k   g  k  v  l

                                          BsaJI
                                          BseDI
                                          BssECI
                          BciVI
                          BfuI
2461  gcgcgacata ttgtcccgta tccgccaggg attcctatta ttttcaaagg agaaacaata
      cgcgctgtat aacagggcat aggcggtccc taaggataat aaaagtttcc tctttgttat
      >.........................SAOUHSC_00450.........................>
        a  r  h   i  v  p   y  p  p  g   i  p  i   i  f  k   g  e  t  i

2521  actgaaaata tgatagaatt ggtaaatgaa tatctggaaa ctggaatgat agttgaagga
      tgacttttat actatcttaa ccatttactt atagaccttt gaccttacta tcaacttcct
      >.........................SAOUHSC_00450.........................>
        t  e  n   m  i  e   l  v  n  e   y  l  e   t  g  m   i  v  e  g

2581  attaaaaata ataaaatttt agttgaggat gaataaaatg tcagctttta taacttttga
      taatttttat tattttaaaa tcaactccta cttatttttac agtcgaaaat attgaaaact
                                        StopStart
      >............SAOUHSC_00450...........>>
        i  k  n   n  k  i   l  v  e  d   e  -
                                          >>.........tmk..........>
                                            m   s  a  f   i  t  f

      ApaI
      BshFI
      BsnI
      BspANI
      BsuRI
      HaeIII
      PhoI
      Bsp120I
      DraII
      EcoO109I
      PspOMI
2641  gggcccagaa ggctctggaa aaacaactgt aattaatgaa gtttaccata gattagtaaa
      cccgggtctt ccgagacctt tttgttgaca ttaattactt caaatggtat ctaatcattt
      >.............................tmk.............................>
      e  g  p  e   g  s  g   k  t  t   v  i  n  e   v  y  h   r  l  v

                                      MabI
                                      SexAI
2701  agattatgat gtcattatga ctagagaacc aggtggtgtt cctactggtg aagaaatacg
      tctaatacta cagtaatact gatctcttgg tccaccacaa ggatgaccac ttctttatgc
      >.............................tmk.............................>
      k  d  y  d   v  i  m   t  r  e   p  g  g  v   p  t  g   e  e  i

2761  taaaattgta ttagaaggca atgatatgga cattagaact gaagcaatgt tatttgctgc
      attttaacat aatcttccgt tactatacct gtaatcttga cttcgttaca ataaacgacg
      >.............................tmk.............................>
      r  k  i  v   l  e  g   n  d  m   d  i  r  t   e  a  m   l  f  a

      XbaI
2821  atctagaaga gaacatcttg tattaaaggt cataccagct ttaaaagaag gtaaggttgt
      tagatcttct cttgtagaac ataatttcca gtatggtcga aattttcttc cattccaaca
      >.............................tmk.............................>
      a  s  r  r   e  h  l   v  l  k   v  i  p  a   l  k  e   g  k  v

2881  gttgtgtgat cgctatatcg atagttcatt agcttatcaa ggttatgcta gagggattgg
      caacacacta gcgatatagc tatcaagtaa tcgaatagtt ccaatacgat ctccctaacc
      >.............................tmk.............................>
      v  l  c  d   r  y  i   d  s  s   l  a  y  q   g  y  a   r  g  i
```

```
2941  cgttgaagaa gtaagagcat taaacgaatt tgcaataaat ggattatatc cagacttgac
      gcaacttctt cattctcgta atttgcttaa acgttattta cctaatatag gtctgaactg
      >.............................tmk.............................>
      g  v  e  e   v  r  a   l  n  e   f  a  i  n   g  l  y   p  d  l

                                      Bsp68I
                                      BtuMI
                                      NruI
3001  gatttattta aatgttagtg ctgaagtagg tcgcgaacgt attattaaaa attcaagaga
      ctaaataaat ttacaatcac gacttcatcc agcgcttgca taataatttt taagttctct
      >.............................tmk.............................>
      t  i  y  l   n  v  s   a  e  v   g  r  e  r   i  i  k   n  s  r

                                                                BstEII
                                                                BstPI
                                                                Eco91I
                                                                EcoO65I
                                                                PspEI
3061  tcaaaataga ttagatcaag aagatttaaa gtttcacgaa aaagtaattg aaggttacca
      agttttatct aatctagttc ttctaaattt caaagtgctt tttcattaac ttccaatggt
      >.............................tmk.............................>
      d  q  n  r   l  d  q   e  d  l   k  f  h  e   k  v  i   e  g  y

3121  agaaatcatt cataatgaat cacaacggtt caaaagcgtt aatgcagatc aacctcttga
      tctttagtaa gtattactta gtgttgccaa gttttcgcaa ttacgtctag ttggagaact
      >.............................tmk.............................>
      q  e  i  i   h  n  e   s  q  r   f  k  s  v   n  a  d   q  p  l

3181  aaatgttgtt gaagacacgt atcaaactat catcaaatat ttagaaaaga tatgatataa
      tttacaacaa cttctgtgca tagtttgata gtagtttata aatcttttct atactatatt
                                                             Stop -10
      >...........................tmk...........................>>
      e  n  v  v   e  d  t   y  q  t   i  i  k  y   l  e  k   i  -

3241  ttgttagaag aggtgttata aaatgaaaat gattatagcg atcgtacaag atcaagatag
      aacaatcttc tccacaatat tttacttta ctaatatcgc tagcatgttc tagttctatc
                            Start
                             >>.............PII-like.................>
                               m  k   m  i  i  a   i  v  q   d  q  d

3301  tcaggaactt gcagatcaac ttgttaaaaa taactttaga gcaacaaaat tggcaacaac
      agtccttgaa cgtctagttg aacaattttt attgaaatct cgttgtttta accgttgttg
      >...........................PII-like.............................>
      s  q  e  l   a  d  q   l  v  k   n  n  f  r   a  t  k   l  a  t

                      AccBSI
                      BsrBI
                      MbiI
3361  aggtgggttt ttaagagcgg gtaatacaac attcttatgt ggtgtcaatg atgaccgtgt
      tccacccaaa aattctcgcc cattatgttg taagaataca ccacagttac tactggcaca
      >...........................PII-like.............................>
      t  g  g  f   l  r  a   g  n  t   t  f  l  c   g  v  n   d  d  r

3421  agatgaaata ttgtctgtga ttaatcaaac gtgtggtaat agagaacagt tggtttcacc
      tctactttat aacagacact aattagtttg cacaccatta tctcttgtca accaaagtgg
      >...........................PII-like.............................>
      v  d  e  i   l  s  v   i  n  q   t  c  g  n   r  e  q   l  v  s

                                  BtsI
                  BstENI
                  EcoNI
                  XagI
3481  tattacacct atgggaggca gtgcggattc gtacattcca tatccagttg aagttgaagt
      ataatgtgga taccctccgt cacgcctaag catgtaaggt ataggtcaac ttcaacttca
      >...........................PII-like.............................>
      p  i  t  p   m  g  g   s  a  d   s  y  i  p   y  p  v   e  v  e
```

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```
3541  tggcggtgct actgtatttg ttatgccagt tgatgcattc catcaatttt aattctataa
      accgccacga tgacataaac aatacggtca actacgtaag gtagttaaaa ttaagatatt
                                                            Stop
      >.....PII-like.....>>
      v  g  g  a   t  v  f   v  m  p   v  d  a  f   h  q  f   -

3601  tacaatcatc aattagagat acttaaaata gtgtattaat aagttattaa caattttggg
      atgttagtag ttaatctcta tgaatttttat cacataatta ttcaataatt gttaaaaccc

                    AhlI
                    BcuI
                    SpeI
3661  ttgcttgact gcgactagtt cagatgccaa tagatttgat ttttgtggtt ctaaaaataa
      aacgaactga cgctgatcaa gtctacggtt atctaaacta aaaacaccaa gatttttatt
                                              SAOUHSC_00453 <<.....<
                                                            -  f  y

3721  tcacaaatca tggtcgctat tgttgcagta attagttgct ctttggcaac ctttttatat
      agtgtttagt accagcgata acaacgtcat taatcaacga gaaaccgttg gaaaaatata
      <.....SAOUHSC_00453.....<
       d  c  i   m  t  a  i   t  a  t   i  l  q   e  k  a  v   k  k  y

3781  aaaagcaaaa gggagtttgt aatgaatgga tgaacagcaa caattgacga atgcatatca
      ttttcgtttt ccctcaaaca ttacttacct acttgtcgtt gttaactgct tacgtatagt
      <.....SAOUHSC_00453.....<
       l  l  l   l  s  n  t   i  f  p   h  v  a   v  i  s  s   h  m  d

                    BbuI
                    PaeI
                    SphI
3841  ttcaaataaa ttatcgcatg cctatttatt tgaaggtgat gatgcacaaa cgatgaaaca
      aagtttattt aatagcgtac ggataaataa acttccacta ctacgtgttt gctactttgt
      <.....SAOUHSC_00453.....<<
       n  l  y   i  i  a  h   r  n  i   q  l  h   h  h  v
                          DNA polymerase III subunit delta' >>.....>
                                                            m  k

3901  agttgcgatt aattttgcaa agcttatttt atgtcaaaca gatagtcaat gtgaaacaaa
      tcaacgctaa ttaaaacgtt tcgaataaaa tacagtttgt ctatcagtta cactttgttt
      >.....DNA polymerase III subunit delta'.....>
      q  v  a  i   n  f  a   k  l  i   l  c  q  t   d  s  q   c  e  t

3961  ggttagtaca tataatcatc cagactttat gtatatatca acaactgaga atgcaattaa
      ccaatcatgt atattagtag gtctgaaata catatatagt tgttgactct tacgttaatt
      >.....DNA polymerase III subunit delta'.....>
      k  v  s  t   y  n  h   p  d  f   m  y  i  s   t  t  e   n  a  i

4021  gaaagaacaa gttgaacaac ttgtgcgtca tatgaatcaa cttcctatag aaagcacaaa
      ctttcttgtt caacttgttg aacacgcagt atacttagtt gaaggatatc tttcgtgttt
      >.....DNA polymerase III subunit delta'.....>
      k  k  e  q   v  e  q   l  v  r   h  m  n  q   l  p  i   e  s  t

4081  taaagtgtac atcattgaag actttgaaaa gttaactgtt caaggggaaa acagtatctt
      atttcacatg tagtaacttc tgaaactttt caattgacaa gttccccttt tgtcatagaa
      >.....DNA polymerase III subunit delta'.....>
      n  k  v  y   i  i  e   d  f  e   k  l  t  v   q  g  e   n  s  i

                                                    AccI
                                                    FblI
                          BsaWI                     XmiI
4141  gaaatttctt gaagaaccac cggacaatac gattgctatt ttattgtcta caaaacctga
      ctttaaagaa cttcttggtg gcctgttatg ctaacgataa aataacagat gttttggact
      >.....DNA polymerase III subunit delta'.....>
      l  k  f  l   e  e  p   p  d  n   t  i  a  i   l  l  s   t  k  p
```

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```
4201  gcaaatttta gacacaatcc attcaaggtg tcagcatgta tatttcaagc ctattgataa
      cgtttaaaat ctgtgttagg taagttccac agtcgtacat ataaagttcg gataactatt
      >..............DNA polymerase III subunit delta'..............>
      e  q  i  l   d  t  i   h  s  r   c  q  h  v   y  f  k   p  i  d

                                                          AlwNI
                                                          CaiI
4261  agaaaagttt ataaatagat tagttgaaca aaacatgtct aagccagtag ctgaaatgat
      tcttttcaaa tatttatcta atcaacttgt tttgtacaga ttcggtcatc gactttacta
      >..............DNA polymerase III subunit delta'..............>
      k  e  k  f   i  n  r   l  v  e   q  n  m  s   k  p  v   a  e  m

      AssI
      BmcAI
      ScaI
      ZrmI
4321  tagtacttat actacgcaaa tagataatgc aatggcttta aatgaagaat ttgatttatt
      atcatgaata tgatgcgttt atctattacg ttaccgaaat ttacttctta aactaaataa
      >..............DNA polymerase III subunit delta'..............>
      i  s  t  y   t  t  q   i  d  n   a  m  a  l   n  e  e   f  d  l

4381  agcattaagg aaatcagtta tacgttggtg tgaattgttg cttactaata agccaatggc
      tcgtaattcc tttagtcaat atgcaaccac acttaacaac gaatgattat tcggttaccg
      >..............DNA polymerase III subunit delta'..............>
      l  a  l  r   k  s  v   i  r  w   c  e  l  l   l  t  n   k  p  m

4441  acttataggt attattgatt tattgaaaca ggctaaaaat aaaaaactgc aatctttaac
      tgaatatcca taataactaa ataactttgt ccgattttta ttttttgacg ttagaaattg
      >..............DNA polymerase III subunit delta'..............>
      a  l  i  g   i  i  d   l  l  k   q  a  k  n   k  k  l   q  s  l

                                  AsuII
                                  Bpu14I
                                  Bsp119I
                                  BspT104I
                                  BstBI
                                  Csp45I
                                  NspV
                                  SfuI
4501  tattgcagct gtgaatggtt tcttcgaaga tatcatacat acaaaggtaa atgtagagga
      ataacgtcga cacttaccaa agaagcttct atagtatgta tgtttccatt tacatctcct
      >..............DNA polymerase III subunit delta'..............>
      t  i  a  a   v  n  g   f  f  e   d  i  i  h   t  k  v   n  v  e

                                                      Acc16I
                                                      AviII
                                                      FspI
                                                      NsbI
4561  taaacaaata tatagtgatt taaaaaatga tattgatcaa tatgcgcaaa agttgtcgtt
      atttgtttat atatcactaa atttttttact ataactagtt atacgcgttt tcaacagcaa
      >..............DNA polymerase III subunit delta'..............>
      d  k  q  i   y  s  d   l  k  n   d  i  d  q   y  a  q   k  l  s

4621  taatcaatta attttgatgt ttgatcaact gacggaagca cataagaaat tgaatcaaaa
      attagttaat taaaactaca aactagttga ctgccttcgt gtattcttta acttagtttt
      >..............DNA polymerase III subunit delta'..............>
      f  n  q  l   i  l  m   f  d  q   l  t  e  a   h  k  k   l  n  q

4681  tgtaaatcca acgcttgtat ttgaacaaat cgtaattaag ggtgtgagtt agatgccaaa
      acatttaggt tgcgaacata aacttgttta gcattaattc ccacactcaa tctacggttt
      >..........DNA polymerase III subunit delta'..........>>
      n  v  n  p   t  l  v   f  e  q   i  v  i  k   g  v  s   -
                                                  SAOUHSC_00455 >>.....>
                                                                m  p
```

```
 4741  tgtaataggt gttcagtttc aaaaagcggg aaaattagaa tattatacac ctaatgatat
       acattatcca caagtcaaag tttttcgccc ttttaatctt ataatatgtg gattactata
       >.......................SAOUHSC_00455.......................>
       n  v  i  g   v  q  f   q  k  a   g  k  l  e   y  y  t   p  n  d

                                BfiI
                               BmrI
                               BmuI
 4801  acaagtagat atagaagact gggtagttgt cgaatctaaa agaggcatag agataggtat
       tgttcatcta tatcttctga cccatcaaca gcttagattt tctccgtatc tctatccata
       >.......................SAOUHSC_00455.......................>
       i  q  v  d   i  e  d   w  v  v   v  e  s  k   r  g  i   e  i  g

 4861  tgttaaaaat ccattaatgg atattgctga agaggatgtt gtgttacctc ttaaaaatat
       acaattttta ggtaattacc tataacgact tctcctacaa cacaatggag aatttttata
       >.......................SAOUHSC_00455.......................>
       i  v  k  n   p  l  m   d  i  a   e  e  d  v   v  l  p   l  k  n

 4921  tattcgcatt gctgatgaca aagatattga taaatttaat tgtaatgaac gagatgctga
       ataagcgtaa cgactactgt ttctataact atttaaatta acattacttg ctctacgact
       >.......................SAOUHSC_00455.......................>
       i  i  r  i   a  d  d   k  d  i   d  k  f  n   c  n  e   r  d  a

 4981  aaatgcatta atactatgta aagacattgt aagagaacaa ggtttggaca tgcgtttagt
       tttacgtaat tatgatacat ttctgtaaca ttctcttgtt ccaaacctgt acgcaaatca
       >.......................SAOUHSC_00455.......................>
       e  n  a  l   i  l  c   k  d  i   v  r  e  q   g  l  d   m  r  l

 5041  caattgcgaa tatacattag ataaatcgaa agttatttt aattttacgg cggatgatcg
       gttaacgctt atatgtaatc tatttagctt tcaataaaaa ttaaaatgcc gcctactagc
       >.......................SAOUHSC_00455.......................>
       v  n  c  e   y  t  l   d  k  s   k  v  i  f   n  f  t   a  d  d

 5101  tattgatttt agaaaattag taaaaatatt agcgcaacat ttaaaaacac gtatcgagtt
       ataactaaaa tcttttaatc atttttataa tcgcgttgta aatttttgtg catagctcaa
       >.......................SAOUHSC_00455.......................>
       r  i  d  f   r  k  l   v  k  i   l  a  q  h   l  k  t   r  i  e

                                                          AvaII
                                                         Bme18I
                                                         Eco47I
                                                          SinI
                                                        VpaK11BI
 5161  gagacaaatt ggtgtaaggg atgaagccaa attgcttggc ggtatcggac cttgtggtag
       ctctgtttaa ccacattccc tacttcggtt taacgaaccg ccatagcctg gaacaccatc
       >.......................SAOUHSC_00455.......................>
       l  r  q  i   g  v  r   d  e  a   k  l  l  g   g  i  g   p  c  g

 5221  gtcgttatgt tgttctacat ttttagggga ttttgaacca gtatcgatta agatggctaa
       cagcaataca acaagatgta aaaatcccct aaaacttggt catagctaat tctaccgatt
       >.......................SAOUHSC_00455.......................>
       r  s  l  c   c  s  t   f  l  g   d  f  e  p   v  s  i   k  m  a

 5281  ggatcaaaat ttatcattaa atccaactaa aatttctggt gcatgtggtc gtttgatgtg
       cctagtttta aatagtaatt taggttgatt ttaaagacca cgtacaccag caaactacac
       >.......................SAOUHSC_00455.......................>
       k  d  q  n   l  s  l   n  p  t   k  i  s  g   a  c  g   r  l  m

 5341  ttgtttaaaa tatgaaaatg actattatga ggaagtacgt gcacaattac ctgatattgg
       aacaaatttt atacttttac tgataatact ccttcatgca cgtgttaatg gactataacc
       >.......................SAOUHSC_00455.......................>
       c  c  l  k   y  e  n   d  y  y   e  e  v  r   a  q  l   p  d  i
```

```
5401  tgaagcaatt gaaacgcctg atggtaacgg gaaagtagtt gctttaaata tattagacat
      acttcgttaa ctttgcggac taccattgcc ctttcatcaa cgaaatttat ataatctgta
      >.........................SAOUHSC_00455.........................>
      g  e  a  i   e  t  p   d  g  n   g  k  v  v   a  l  n   i  l  d

5461  ttctatgcag gtgaagcttg agggacatga acagccactt gaatataaat tagaagaaat
      aagatacgtc cacttcgaac tccctgtact tgtcggtgaa cttatatttа atcttcttta
      >.........................SAOUHSC_00455.........................>
      i  s  m  q   v  k  l   e  g  h   e  q  p  l   e  y  k   l  e  e

5521  agaaactatg cattaaggag gcattattac atttggatcg caatgaaata tttgaaaaaa
      tctttgatac gtaattcctc cgtaataatg taaacctagc gttactttat aaacttttt
                      SD
      >.SAOUHSC_00455>>
      i  e  t  m   h  -
                                        >>...........YabA............>
                                          l  d   r  n  e  i   f  e  k

5581  taatgcgttt agaaatgaat gtcaatcaac tttcaaagga aacttcagaa ttaaaggcac
      attacgcaaa tctttactta cagttagttg aaagtttcct ttgaagtctt aatttccgtg
      >.............................YabA.............................>
       i  m  r   l  e  m  n   v  n  q   l  s  k   e  t  s  e   l  k  a

                                        Bsp143II
                                        BstH2I
                                        HaeII
                                        AfeI
                                        Aor51HI
                                        Eco47III
5641  ttgcagttga attagtagaa gaaaatgtag cgcttcaact tgaaaatgat aatttgaaaa
      aacgtcaact taatcatctt cttttacatc gcgaagttga acttttacta ttaaacttttt
      >.............................YabA.............................>
       l  a  v   e  l  v  e   e  n  v   a  l  q   l  e  n  d   n  l  k

                                                          EcoRI
5701  aggtgttggg caatgatgaa ccaactacta ttgatactgc gaattcaaaa ccagcaaaag
      tccacaaccc gttactactt ggttgatgat aactatgacg cttaagtttt ggtcgttttc
      >.............................YabA.............................>
       k  v  l   g  n  d  e   p  t  t   i  d  t   a  n  s  k   p  a  k

5761  ctgtgaaaaa gccattacca agtaaagata atttggctat attgtatgga gaaggatttc
      gacacttttt cggtaatggt tcatttctat taaaccgata taacatacct cttcctaaag
      >.............................YabA.............................>
       a  v  k   k  p  l  p   s  k  d   n  l  a   i  l  y  g   e  g  f

5821  atatttgtaa aggcgaatta tttggaaaac atcgacatgg tgaagattgt ctgttctgtt
      tataaacatt tccgcttaat aaaccttttg tagctgtacc acttctaaca gacaagacaa
      >.............................YabA.............................>
       h  i  c   k  g  e  l   f  g  k   h  r  h   g  e  d  c   l  f  c

5881  tagaagtttt aagtgattaa tcaagcacac tcaaatagtg ttataattat aaatgaatat
      atcttcaaaa ttcactaatt agttcgtgtg agtttatcac aatattaata tttacttata
      >.......YabA.......>>
       l  e  v   l  s  d  -

5941  ggtttggata agtctgagac aatgcatgtt tcaggcttta attgtgtata aagttttggt
      ccaaacctat tcagactctg ttacgtacaa agtccgaaat taacacatat ttcaaaacca

6001  gattgcataa gagatggcgg tactaaatgt tattattaag tgtgcacgca gtatcattag
      ctaacgtatt ctctaccgcc atgatttaca ataataattc acacgtgcgt catagtaatc

6061  ttataaaatg tagctgttaa aagtcaaaaa tacatcgaat gtagttaggc atataatata
      aatattttac atcgacaatt ttcagttttt atgtagctta catcaatccg tatattatat
```

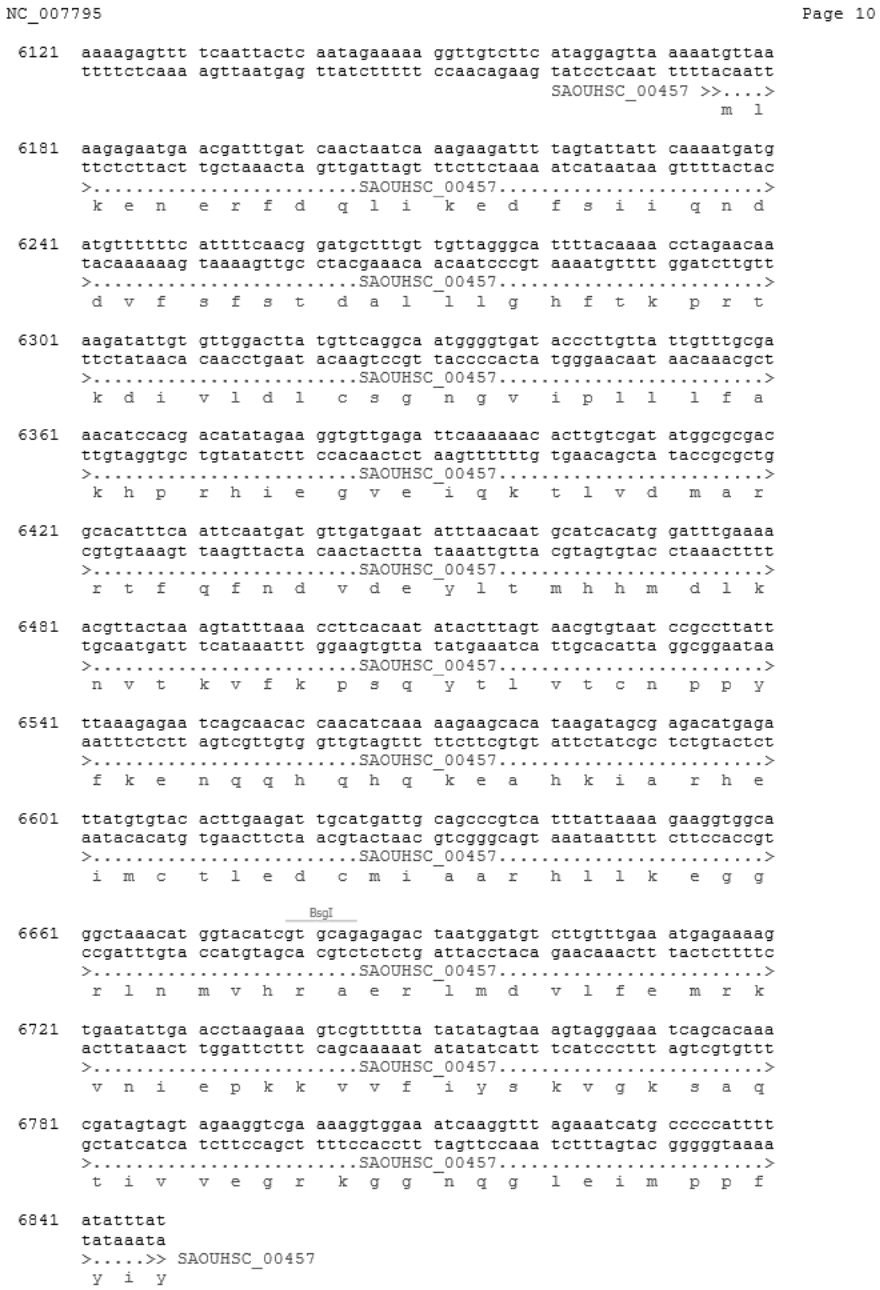

Figure 34. Nucleotide sequence of SAOUHSC_00452 encoding for putative *PII-like* from *S. aureus*

The deviated amino acid sequences of putative *PII-like* in *S. aureus* are illustrated below and above the nucleotide sequences.

G. Appendix

G.1 Index of Tables

G.2 Index of Figures

H. REFERENCES

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