Activity spectrum, in vivo efficacy of albomycin and its use in characterization of hydroxamate iron transport in *Streptococcus pneumoniae*

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1. Introduction

1.1. The boundary of bacteria

Bacteria are very small, mostly living in unicellular form with size ranging from 0.5 to 1.0 μm in diameter. That brings very high surface to volume ratio and the accompanied challenge to maintain the cell shape with a defined system boundary in an ever-changing environment. The solution came as a combination of impermeable lipid bilayer membrane/s and a rigid layer composed of highly cross-linked structure, called murein. The building block of murein is peptidoglycan, very unique to prokaryotes. Quite evidently an array of antibacterials like penicillin, cephalosporin, bacitracin which inhibit synthesis of peptidoglycan layer is long been proven very effective to clear bacterial infections in humans without much ill side effects.

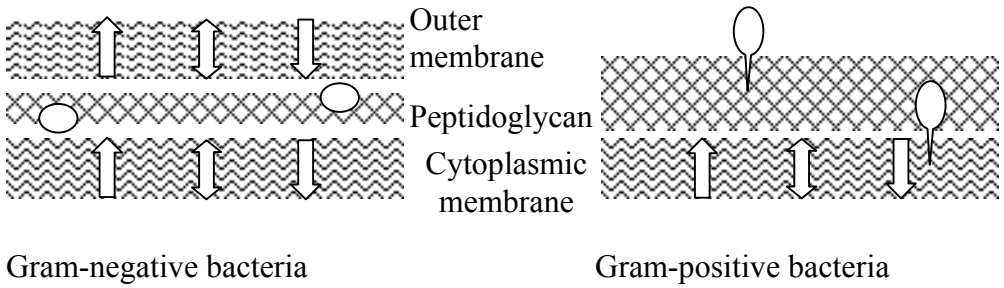


Figure 1 Schematic drawing to compare general features of cellular boundary in gram negative and positive bacteria.

Peptidoglycan is very thick and rigid in case of gram-positive bacteria and so sufficient to maintain cell pressure. Whereas to compensate the less complex peptidoglycan layer gram-negative bacteria employ another bilayer boundary called outer membrane. Murein gives the mechanical strength to maintain the steady shape but separated from the cellular constituents by cytoplasmic membrane which is the immediate layer next to cell mass. While the most cross-linked peptidoglycan is porous to even larger macromolecules up to 40 kd, cytoplasmic and outer membrane pose a strong hydrophobic barrier to penetration by hydrophilic and charged molecules (Bidnenko *et al.*, 1998; Nakae and Nikaido, 1975; Sara and Sleytr, 1987; Scherrer and Gerhardt, 1964, 1971). Uptake of nutrients from the environment and waste disposal cannot effectively be done by only

passive diffusion. These membrane structures host multiple large protein complexes to facilitate directional diffusion or at times active transport to import nutrients of interest or to dispose waste materials.

1.2. Passage through membrane barriers

Although impermeable to large molecules the outer membrane allows passage of small molecules, generally smaller than 600 da in size like oligosaccharides, nucleosides, amino acids, small peptides irrespective of polarity via special protein complexes called porins (Hancock, 1997; Nikaido, 1988; Zimmermann and Rosselet, 1977). Porins are abundant in gram-negative species though the permissible sizes can vary from species to species, as reported in *Pseudomonas*, which can allow bigger molecules to pass through (Aires *et al.*, 1999; Hancock *et al.*, 1979; Nikaido, 1994; Yoshimura and Nikaido, 1982). Other than this facilitated diffusion, multi-component protein complexes involved in substrate specific active transport across the membrane of relatively scarce nutrients, plays a key role in bacterial survival and fitness. Role of active transport have been demonstrated for virtually all types of solutes including sugars, amino acids, peptides, nucleosides, metal and organic ions (Benz *et al.*, 1993; Braun and Killmann, 1999; Dean *et al.*, 1989; Hall *et al.*, 1997; Juillard *et al.*, 1998; Kaback, 2005). Given the different membrane organization, the positioning and number of necessary components for active transport differs considerably between gram positive and negative bacteria (Andrade *et al.*, 2002; Braun, 2003; Claverys, 2001).

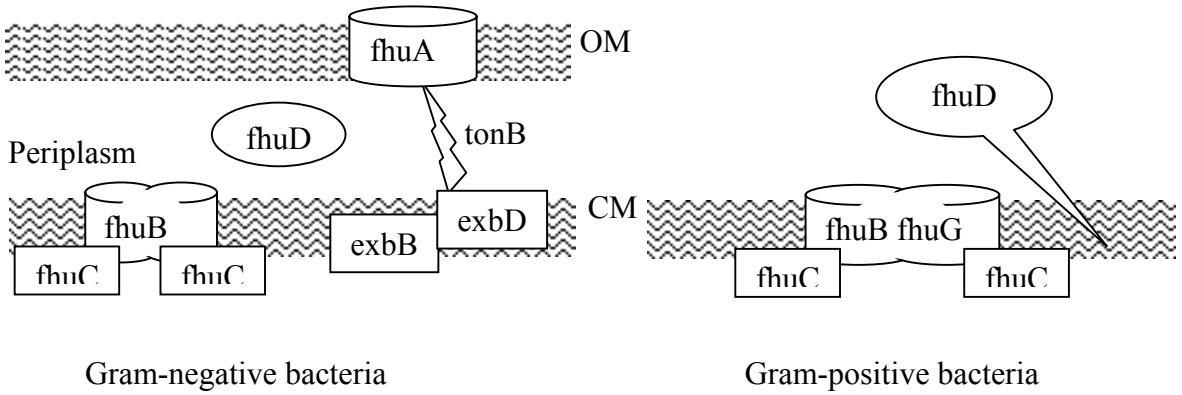


Figure 2. Components of active Fe^{3+} hydroxamate transport system in gram negative and gram-positive bacteria.

In gram-negative species large proteins in outer membrane, energized by the TonB-ExbB-ExbD complex, serve as the receptor and transmembrane transporter to deliver collected substrates to periplasm (Chimento *et al.*, 2005; Ferguson and Deisenhofer, 2002; Letain and Postle, 1997; Postle and Kadner, 2003). There free periplasmic substrate binding proteins collect the specific substrates and deliver it to the cognate transmembrane permease/s – ATPase protein complex sitting in the cytoplasmic membrane to finally send the substrates to the cytoplasm (Braun and Braun, 2002; Dean *et al.*, 1992). In gram-positive bacteria a necessity for outer membrane receptors is omitted. Instead, substrates can diffuse through the porous murein layer where substrate binding protein (equivalent to gram negative periplasmic binding protein) anchored to cytoplasmic membrane via a covalently attached lipid molecule, catches the substrate and specifically delivers it to the cognate permease-ATPase complexes for bringing the bound substrate into the cytoplasm (Alloing *et al.*, 1994; Bouvier *et al.*, 2000; Gilson *et al.*, 1988; Hardham *et al.*, 1997; Sutcliffe and Harrington, 2004).

1.3. Availability and importance of iron to biological systems

Iron is a vital nutrient for all living organism including bacteria with a very few known exceptions like lactobacilli and *Borrelia burgdorferi*, which require no or less iron for survival (Atkin and Neilands, 1968; Neilands, 1976; Posey and Gherardini, 2000). Iron is essential as cofactor in driving a variety of vital cellular functions; most importantly for Fe-S redox proteins, cytochrome oxidases, TCA cycle metallo-enzymes, aerobic type ribonucleotide reductases, dehydratases, catalases and others (Atkin and Neilands, 1968; Neilands, 1976). Chemicals like cyanide or azide, which interfere with the functionality of iron containing enzymes, are notoriously lethal for almost all living cells (Anraku *et al.*, 1975; Chen *et al.*, 1999; Hansen and Nicholls, 1978; Tsubaki *et al.*, 1999; Viola *et al.*, 1996; Yamanaka *et al.*, 1985). Though iron is the 2nd most abundant metal in earth crust, presence of uncomplexed iron in aerobic environment is surprisingly low. At physiological pH in aqueous environment Fe^{+3} forms large insoluble oxyhydroxide polymer of composition $(FeOOH)_n$. In aqueous solution the free iron concentration is of about 10^{-9} M, which is much less than the concentration (10^{-7} M) required to support optimal growth for most bacterial species (Braun, 1997, 2001, 2003). For pathogenic organisms the situation is even worse, as extra cellular fluids of human hosts allow free iron at the range of 10^{-24} M (Braun, 2005). To successfully compete to scavenge iron from such hostile environment, different

microorganisms have developed a variety of strategies to acquire iron to a level to support growth. Study of different iron acquisition mechanism and their role in survival and virulence of bacteria has long been a major thrust in microbiology (Angerer *et al.*, 1992; Ardon *et al.*, 2001; Baumler *et al.*, 1993; Berner and Winkelmann, 1990; Brown and Holden, 2002; Dellagi *et al.*, 1998; Evans *et al.*, 1986; Griffiths *et al.*, 1984; Hantke and Braun, 1975; Leong and Neilands, 1976; Mahren *et al.*, 2005; Peters and Warren, 1968; Sokol *et al.*, 1999; Yancey and Finkelstein, 1981).

1.4. Solubilizing with siderophores

A very effective and thus widely distributed strategy is to recruit a group of structurally different low molecular weight hydrophilic iron chelating compounds termed siderophore to scavenge iron. At the oxidised state freely distributed soluble siderophore molecules snatch the iron from the environment with very high affinity. The iron loaded siderophore are picked from the environment and transported inside the energized cell by siderophore specific active transport systems. Once inside the cell iron bound siderophores are reduced to release the iron for further use by the cell. Most fungi with the exception of budding and fission yeast synthesize and secrete siderophore in the environment and utilize them for iron acquisition. While many species of bacteria can synthesize specific siderophores and utilize them, quite frequently they can also cheat on the siderophores produced by other bacterial and even fungal species. *Escherichia coli*, a well characterised widely distributed gram negative species carries a burden of at least 7 different iron transport systems including for siderophores of fungal origin, explaining the importance of iron acquisition on the fitness of bacteria in a competitive niche (Alderete *et al.*, 2004; Avendano-Herrera *et al.*, 2005; Bahrami and Niven, 2005; Braun, 2005; Dale *et al.*, 2004; Dashper *et al.*, 2004; Ferreras *et al.*, 2005; Gancz *et al.*, 2006; Haag *et al.*, 1993; Harvie and Ellar, 2005; Heesemann *et al.*, 1993; Holmes *et al.*, 2005; Kustos *et al.*, 2005; Lee and Han, 2006; Palyada *et al.*, 2004; Perkins-Balding *et al.*, 2003; Perkins-Balding *et al.*, 2004; Ratledge, 2004; Reid and Kirov, 2004; Russo *et al.*, 2003; Sabri *et al.*, 2006; Schaible and Kaufmann, 2004; Schrettl *et al.*, 2004; Skaar *et al.*, 2004; Snyder *et al.*, 2004; Speziali *et al.*, 2006; Visser *et al.*, 2004).

1.5. Emerging antibiotic resistance

The ability of antibiotics to stop an infection depends on killing or halting the growth of bacteria. Introduction of antimicrobial drugs, most notably penicillin was thought to be the start of the end of bacterial infection. From 1945 to late 1980s, rapid discovery of new classes of antimicrobial agents following penicillin opened up a new era of lifesaving medical treatment. However in the '80s and '90s there's almost no new classes of antibacterials reached market, but rather improvement within the present classes. Alarmingly, there were reports of penicillin resistance within one year of its introduction (Rammelkamp, 1942) but the pace of antibiotic development was going faster than the development and spread of resistance among pathogenic bacterial species. Success stories of the 'wonder bullets' largely undermined the likely response in the microbial community over time while half of the large U.S. and Japanese pharmaceutical companies either reduced or abandoned programs for further discovery of anti-infectives (Alanis, 2005; Barrett and Barrett, 2003; Barrett, 2005; Bassetti *et al.*, 2002; Boggs and Miller, 2004; Dowell, 2004; Norrby *et al.*, 2005; Projan, 2003; Projan and Shlaes, 2004; Reed *et al.*, 2002; Shlaes, 2003; Spellberg *et al.*, 2004; Thomson *et al.*, 2004; Wenzel, 2004). Surprisingly, most clinically relevant antibiotics to date originated only from a few soil dwelling bacteria. As a consequence innate microbial resistance spans all known classes of natural and synthetic antibacterial compounds (D'Costa *et al.*, 2006). By the end of the 20th century after only fifty years of usage of antibiotics, dissemination of acquired resistance started becoming a matter of clinical concern and with each passing decade, bacteria that resist not only single but multiple antibiotics, started making some disease situations hard to control. As a common trend virtually all significant infectious bacteria in the world are developing resistance to the antibiotic treatment of choice.

1.6. Can sideromycins do?

Though siderophore dependent iron transport is the most common way to overcome iron limitation in bacteria and fungi, no siderophore dependent iron transport had yet been reported in higher order animals. Theoretically, this provides a gate for targeted delivery of antibacterials to pathogens without affecting the plant or animal host in general. Indeed ''natures antibiotic factory'' is the 1st to come up with a class of conjugates called sideromycins, which exploit such gates to kill competing species in the environment.

Sideromycins are nothing but a conjugate of a siderophore and an antibiotic in a way to use both functionalities. Examples of natural sideromycins are albomycin and salmycin (Fig. 3). Sideromycins have some unique merits and demerits.

They can actively bypass permeability barriers to deliver the drug inside the target cell, irrespective of size and polarity of the antibiotic molecule. Most importantly as they are substrates of high affinity transport systems they can be effective at very low concentration. The delivery of the antibiotic will be target specific without affecting the host system.

On the other hand to satisfy the need of iron most bacteria developed a series of high affinity transporters of iron chelators, which leads to the quick development of *in vitro* resistance by dispensing the targeted transport systems. Apparently, those deficiencies do not affect *in vitro* survival considerably, but *in vivo* situation may well be different.

1.7. Albomycin; a laboratory tool with *in vivo* possibilities

Albomycin is a natural sideromycin produced by some streptomycetes. It can act as a double-edged sword. It scavenges iron from the environment making it unavailable for other species, and / or it will actively deliver the antibiotic into competing species. This characteristic of sideromycins attracted a lot of attention towards its possible medical application (Braun and Braun, 2002; Budzikiewicz, 2001; Demain and Fang, 2000; Dolence *et al.*, 1991; Heinisch *et al.*, 2002; Heinisch *et al.*, 2003; Kinzel *et al.*, 1998; Kustos *et al.*, 2005; Miller *et al.*, 1991; Minnick *et al.*, 1992; Mollmann *et al.*, 1998; Nemoto *et al.*, 2002; Poras *et al.*, 1998; Wittmann *et al.*, 2002). Earlier studies on albomycin showed that it is highly active against some gram-positive cocci and a number of gram-negative species including *E. coli*. The transport and release of antibiotic at the target of action was well studied in *E. coli* a long time ago. Very recently from a screening program of bacterial t-RNA synthetase inhibitors, it was found that the antibiotic moiety *in vitro* inhibits seryl t-RNA synthetase from both eukaryotic and prokaryotic representatives (Stefanska *et al.*, 2000). But the penetration of the antibiotic moiety alone is very poor to whole cells. In albomycin the antibiotic moiety is conjugated to a siderophore moiety making it as big as 1045 da, which cannot pass through biological membrane barriers by simple diffusion. To date albomycin sensitivity shows 100% co-relation with the presence of functional hydroxamate specific transport system. This feature of albomycin has been very useful in past at laboratories to dissect hydroxamate transport system in *E. coli*, and *Bacillus subtilis*

(Braun, 1999; Braun *et al.*, 2001; Ferguson *et al.*, 2000; Mademidis and Koster, 1998; Rohrbach *et al.*, 1995; Schneider and Hantke, 1993).

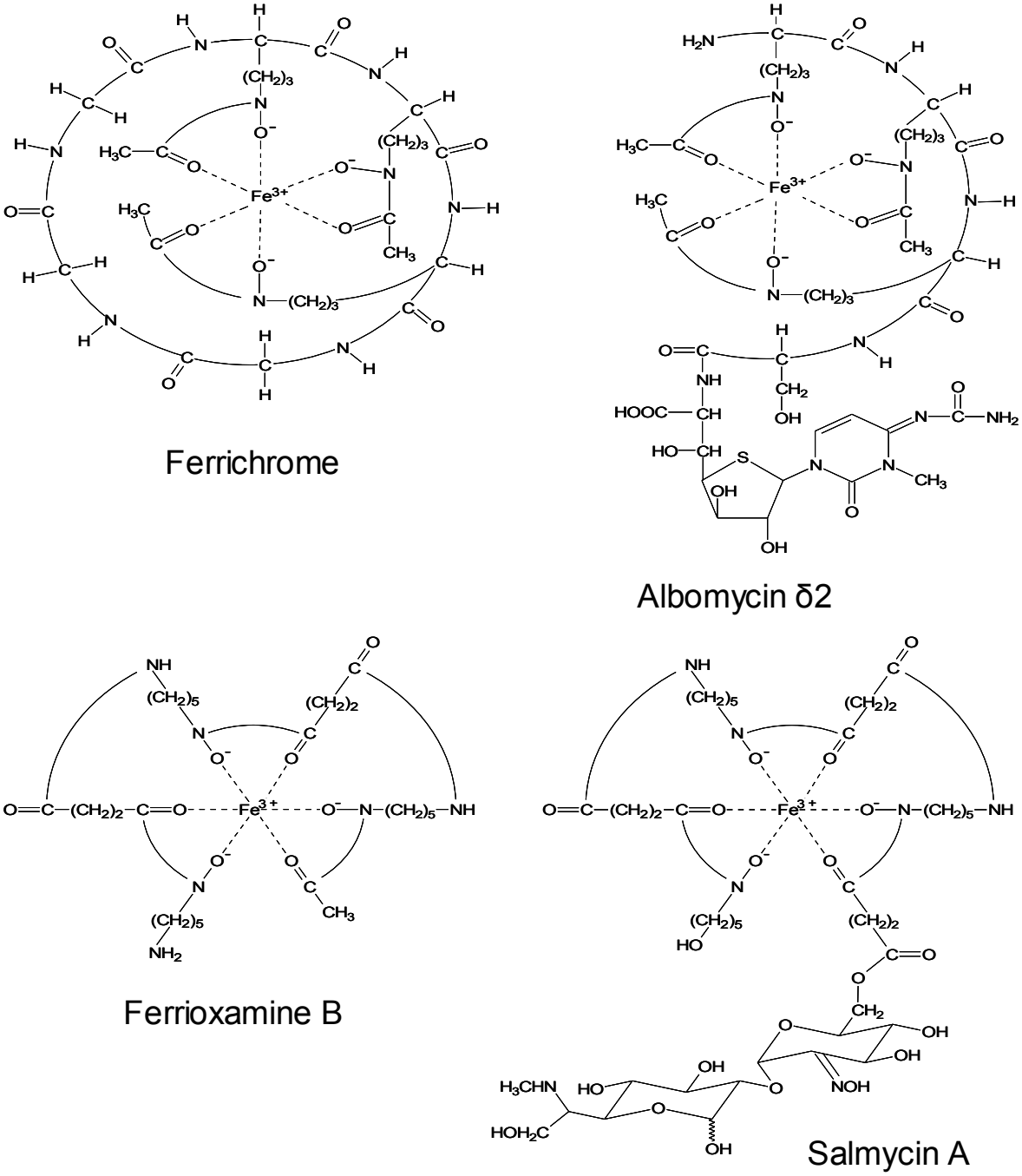


Figure 3 Structure of hydroxamate siderophore prototypes ferrichrome, ferrioxamine B, and the analogous sideromycins albomycin and salmycin respectively.

As of any sideromycin, problem with the potential clinical use of albomycin is, under *in vitro* laboratory condition albomycin treatment always gives rise to spontaneous resistant mutant colonies. But notably all of the spontaneous resistants are defective either in

hydroxamate transport or in the release of antibiotic moiety. A spontaneous resistant mutant with modification in target site has not yet been found (Personal communication Prof. Braun). This can be explained, as unlike eukaryotes, prokaryotes have single copy of each t-RNA synthetases and changes in highly conserved seryl-t-RNA synthetase without compromising protein synthesis could be very difficult to achieve by one step mutation event, if not unlikely. The other major problem albomycin faces is a cost-ineffective production level.

1.8. 'Captain of Death' on the rise

The bacteria, later named *Diplococcus* and renamed as *Streptococcus pneumoniae* were first seen in the airways of individuals who died from pneumonia by Edwin Klebs in 1875 (Klebs, 1875). Sir William Osler, known as "the father of modern medicine," appreciated the morbidity and mortality of pneumonia, describing it as the "captain of the men of death" as early as in 1918. With the advent of penicillin and other antibiotics and intensive care in the twentieth century, mortality from pneumonia dropped precipitously in the developed world. Vaccination of infants against *Haemophilus influenzae* type b, another respiratory tract infection, began in 1988 and led to a dramatic decline in cases shortly thereafter (Adams *et al.*, 1993). Whereas vaccination against *Streptococcus pneumoniae* in adults began in 1977 and in children began in 2000, resulting in decline (Whitney, 2003; Whitney *et al.*, 2003) but also causing a shift in the epidemiology towards nonvaccine serotypes (Hays *et al.*, 2004; Straetemans *et al.*, 2004; Veenhoven *et al.*, 2003; Veenhoven *et al.*, 2004). The root of the problem exists in the fact that more than 90 serotypes of pneumococci exist, and immunization with a given serotype/s did not protect against infection with other serotypes. The current vaccine with maximum coverage protects against 23 serotypes. Despite the fearsome name, pneumococcus is part of the natural flora in nasal cavity of healthy children or adults and is rarely symptomatic. At times by some poorly understood mechanism they turn invasive and can cause many types of infection other than pneumonitis, including acute sinusitis, otitis media, meningitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is the most common cause of bacterial meningitis in adults, and just next to *Haemophilus influenzae* in causing otitis media. There are multiple bacteria and viruses that can individually or in concert cause pneumoniae or oitis media. A recent report on maintaining the balance of community pattern *in vivo* showed components of *H. influenzae*

(but not *S. pneumoniae*) stimulated complement-dependent phagocytic killing of *S. pneumoniae*, whereas *S. pneumoniae* cleared up *H. influenzae* from complement negative environments *per se* (Lysenko *et al.*, 2005). With the ever increasing distribution of drug resistant pneumococci and other pathogens and examples of treatment failures, it became necessary to recharge our arsenal of antimicrobials to fight the stronger bugs of the coming decades (Furuya and Lowy, 2006).

1.9. Objectives of this work

1.9.1. To standardize mini to midi scale fermentation and purification of the antibiotic albomycin to homogeneity.

1.9.2. Scanning the albomycin activity spectrum among common human pathogenic bacterial species.

1.9.3. Identifying genetic determinants for intrinsic resistance shown by the species in sensitivity screen, and evaluate whether that trait can be horizontally transferred to sensitive species.

1.9.4. Identifying major determinants of acquired resistance in sensitive species.

1.9.5. Evaluation of albomycin efficacy in *in vivo* murine infection model and the impact of resistance development in virulence.

2. Materials and methods:

2.1. Materials:

2.1.1. Bacterial Strains

Table 1. Bacterial strains used in the work.

Strain	Parent	Description	Source
***Streptomyces* sp.**			
Tü 6		*Streptomyces griseus*	This institute
DSM 40693		*S. griseus sub sp.griseus*	This institute
ATCC 700974		*Streptomyces* sp.	This institute
ST 03742		*Streptomyces* sp.	This institute
Streptococcus pneumoniae			
D39		capsular serotype 2, the clinical isolate used to demonstrate the genetic function of DNA by Avery, MacLeod, and McCarty.	Dr. Sven Hammerschmidt, Zentrum für Infektionsforschung, Universität Würzburg.
R6	D39	unencapsulated derivative of D39, carrying a 7,504-bp deletion involving nine capsular genes	Prof. Dr. Regine Hakenbeck, Department of Microbiology, Universität Kaiserslautern.
TIGR4		highly virulent, capsular serotype 4, clinical isolate from the blood	Dr. Sven Hammerschmidt, ZINF, Würzburg.
API1	R6	Insertion duplication mutation in *fhuD*	This study
API2	R6	IDM in *spr1687*	This study
API3	R6	IDM in *marR* (metallo regulator)	This study
API4	R6	IDM in ferrochelatase	This study
API5	R6	IDM in *spxB* (pyruvate oxidase)	This study

API6	R6	IDM in *ciaR* (regulator protein)	This study
APD1	R6	Deletion in *fhuD*	This study
APT1	R6	Tn5 insertion in *fhuB*	This study
D39T1	D39	Transposon insertion of APT1 transferred to D39	This study
APT2	R6	Tn5 insertion in *fhuG*	This study
APD1CI	APD1	Erythromycin resistant transposon insertion mixed library (scraped from plate before growing together in broth)	This study
APD1I2	APD1	Deletion in *fhuD* and insertion duplication in *spr1687*	This study
APTEL	R6	Erythromycin resistant random insertion clone library	This study
Escherichia coli			
DH5α		For general cloning purpose.	This institute
SIP401		Lacking fur regulation, high ferrichrome transport, albomycin hypersensitive	This institute
AB2847		Do not produce hydroxamate siderophore aerobactin, thus do not interfere with other siderophore or sideromycin assay.	This institute
C600		*recA*$^+$, *polA*$^+$ strain for replicating and cloning pMV158 derivative plasmids.	This institute
H1717		FURTA strain.	This institute
BL21(DE3)		Contains the T7 RNA polymerase gene under control of the *lacUV5* promoter.	This institute
BL21(DE3) pLysS		These cells have the pLysS plasmid added to them containing T7 lysozyme, a T7 RNA polymerase inhibitor to prevent leaky expression in uninduced cells.	This institute

2.1.2. Plasmids

Table 2. Bacterial plasmids used in the work.

Plasmid	Host	Resistance marker	Description	Source
pJDC9	*E. coli*	Erythromycin	Insertion duplication plasmid, replicates in *E. coli* but not in *S. pneumoniae*	Prof. Dr. Regine Hakenbeck, Kaiserslautern.
pLS101	*E. coli* C600, *S. pneumoniae*	Tetracycline	Rolling circle replication plasmid. Replicates in both *recA*$^+$, *polA*$^+$ *E. coli* and *S. pneumoniae*.	Prof. Dr. Regine Hakenbeck, Kaiserslautern.
pET19b	*E. coli*	Ampicillin	His_6 tag overexpression plasmid vector	EMD Biosciences, Inc
pBR322	*E. coli*	Ampicillin, Tetracycline	General cloning vector	This institute
pET28a	*E. coli*	Kanamycin	His_6 tag over expression plasmid vector	EMD Biosciences, Inc
pMOD3	*E. coli*	Ampicillin	Tn5 transposon construction vector	Epicentre Biotechnologies
pAPT	*E. coli*	Ampicillin, Erythromycin	*ermB* of pJDC9 cloned in pMOD3	This study
pAPID1	*E. coli*	Erythromycin	*fhuD* internal fragment cloned between EcoRI and XbaI of pJDC9	This study
pAPID2	*E. coli*	Erythromycin	*spr1687* internal fragment cloned between EcoRI and BamHI of pJDC9	This study
pAPID3	*E. coli*	Erythromycin	*marR* internal fragment cloned between EcoRI and XbaI of pJDC9	This study
pAPID4	*E. coli*	Erythromycin	Ferrochelatase internal fragment cloned between XbaI and PstI of pJDC9	This study

pAPID5	*E. coli*	Erythromycin	*spxB* internal fragment cloned between KpnI and XbaI of pJDC9	This study
pAPID6	*E. coli*	Erythromycin	*ciaR* internal fragment cloned between SacI and XbaI of pJDC9	This study
pAPBP	*E. coli*	Kanamycin	*fhuD* with N-terminal His-tag cloned into pET-28a between NdeI and BamHI	This study
pAPMR	*E. coli*	Ampicillin	*marR* with N-terminal His-tag cloned into pET-19b between NdeI and BamHI	This study
pAPIC	*E. coli*	Erythromycin	*malM* C-terminal fragment and complete *fhuD* together cloned between SacI and XbaI of pJDC9	This study
pTAP1	*E. coli*	Erythromycin, Tetracycline	digestion fragment (large) from pLS101 cloned in pJDC9 between HindIII and PstI site.	This study
pRCAP1	*E. coli* C600, *S. pneumoniae*	Erythromycin, Tetracycline	PstI digestion fragment from pLS101 inserted in PstI site of pTFAP1, then pJDC9 replication origin destroyed by digestion with HaeII then ligated and transformed into *S. pneumoniae*.	This study
pRCAP2	*E. coli* C600, *S. pneumoniae*	Tetracycline	Partial digestion of pRCAP1 with EcoRI followed by ligation and cloning into *S. pneumoniae*.	This study

2.1.3. Synthetic oligonucleotides

Table 3. Synthetic oligonucleotides used in this work.

Name	Sequence	Modification for
220f	AGCTAGGACGTGAGAAGATGG	nil
224r	GAGACGGTGGTTCGCTAGTCG	nil
934f	AGCTATGGCAGGACTTACAAC	nil
936r	GACCACGGCTTACAAGATCAG	nil
1684f	AGCCAGTGTCCTAGCAGATG	nil
1687r	CTCTTGTCGCTTTGAGCTGAC	nil
NestedF	TGCTTGAACTTGCTTGTTGG	nil
NestedR	TTCAACATTGGCCTTAACCA	nil
934upstF	AGCGTTGAAATGATTGATAAAGGCAA	nil
Sqmod3F	GCCAACGACTACGCACTAGCCAAC	5'CY5
Sqmod3R	GAGCCAATATGCGAGAACACCCGAGAA	5'CY5
M13 rev (-29)	CAGGAAACAGCTATGACC	5'CY5
M13 uni (-21)	TGTAAAACGACGGCCAGT	5'CY5
934fXbaI	AATACTTCTAGAGAGCATGCGCCTG	XbaI
934rEcoRI	GTTGGAATTCATGAGGCTGCTAACG	EcoRI
1687fBamHI	AGCTCGGATCCAACAGAGATAACC	BamHI
1687rEcoRI	TGATTGAATTCCGCCTCCGCTTAG	EcoRI
porBam	TCCTGGATCCTATTTCAAGTCTATTG	BamHI
934SD	GTTTAAGGAGTTCATATGAAGAACAA	NdeI
934EB	TAAGACTGGATCCTGTGTTTATACCGA	BamHI
FCrXba	ATGGTCTAGATGTTCTGTCTGTTC	XbaI
FCfPst	AGTTCTGCAGTCCCTTTATTCCAG	PstI
Marf_Eco	AGGACAAGGAATTCGGCTATCTACTG	EcoRI
Marr_Xba	GGGAGGTTCTAGATTTCAACGAGGAG	XbaI
Ermf_Hind	AGCAAAGCTTGGCGGAAACGTAAAAG	HindIII
Ermr_Bam	TCCTTGGATCCTGTCAGTAGTATACC	BamHI
marSD	AGGAAACATATGACCCCAAACAAAGA	NdeI
marEB	TTTCAAATAGGATCCCACCAAATGAA	BamHI
marfKpn	CTAGCCGTAATCGGTACCTGATCCAA	KpnI

malMFSac	CTTGAGCTCTTTGCTGAGTATA	SacI
malMRNde	GATAACATATGTAGTTGTCTCCTG	NdeI
934EX	TGTGTCTAGACCGAGTATACCTGGA	XbaI
spxfXba	GCTGTCTAGAACAAACGTGTAGCTTA	XbaI
spxrKpn	GAGTAGTGGTACCTACGTCGATTGAG	KpnI
ef1	TGAAGAATTCATCAATCAAGGTACTGG	EcoRI
ciaRFSac	AAGAAGAGCTCTACGAAGCTGAGA	SacI
ciaRR	TCTGCGTCTTAGGCAAAATCACA	nil

2.1.4. Enzymes and Kits

Table 4. Enzymes and Kits used in the work.

Enzymes / Kit	**Purpose**	**Provider**
NucleoBond AX and buffer set A1-3, N1-5	Plasmid extraction	Macherey-Nagel GmbH & Co. KG
NucleoBond AX and buffer set G1-4, N1-5	Genomic DNA extraction	Macherey-Nagel GmbH & Co. KG
Expand High Fidelity PCR System	High fidelity PCR	Roche Diagnostics Corporation
Phusion DNA Polymerase, Buffer and nucleotide set	All purpose PCR	Finnzymes Oy
Multiple restriction enzymes with buffer set.	Restriction digestion	Roche Diagnostics Corporation, New England Biolabs Inc.
T4 DNA Ligase, 10X Buffer with ATP	Regular ligation	Roche Diagnostics Corporation
Fast-Link DNA Ligation Kit	Quick ligation	Epicentre Biotechnologies
NucleoSpin Extract	PCR product and gel purification	Macherey-Nagel GmbH & Co. KG
End-It DNA End-Repair Kit	DNA end repair	Epicentre Biotechnologies
EZ-Tn5 Transposon Construction Vector, EZ-Tn5 Transposase	*In vitro* transposition	Epicentre Biotechnologies
Ni-NTA Agarose	His-tag affinity purification	Qiagen GmbH

2.1.5. Growth media

2.1.5.1. TY medium

Tryptone	8 g
Yeast extract	5 g
NaCl	5 g

Dissolved in 1 l deionised water, then autoclaved.

.

2.1.5.2. NB medium

Nutrient broth	8 g
NaCl	5 g

Dissolved in 1 l deionised water, then autoclaved.

.

2.1.5.3. M9 minimal medium

M9-salt (10 x)	100 ml
Glucose (40%)	10 ml
1 M MgSO4	1 ml
0.1 mM CaCl2	1 ml
Thiamin (1mg/ml)	1 ml

Volume made up to 1 l with deionised water and filter sterilized.

M9-salt (10x)

NaH2PO4, 2 H2O	75 g
KH2PO4	30 g
NH4Cl	10 g
NaCl	5 g

Dissolved in 1 l deionised water, then autoclaved.

2.1.5.4. THY Broth

Todd-Hewitt Bouillon (Roth)	30 g
Yeast extract	5 g

Dissolved in 1 l deionised water, then autoclaved.

For all the above mentioned media 1.5% agar was added before autoclaving if intended to make agar plates.

2.1.5.5. Blood agar plates

Mueller Hinton Broth (Fluka)	23 g
Glucose	1 g
Agar	15 g

Dissolved in 1 l deionised water and autoclaved. After cooling down to ~50 ^{o}C sterile sheep blood was added at the rate of 5 ml per 100 ml media and poured immediately to sterile petri dishes.

2.1.5.6. HA medium

For growing *Streptomyces.*

Glucose	4 g
Malt extract	10 g
Yeast extract	4 g

Dissolved in 1 l deionised water, pH 7.3, then autoclaved.

2.1.5.7. Albomycin production medium

Starch	20 g
L-ornithine-HCl	5 g
KH_2PO_4	1.8 g
$Na_2HPO_4.2H_2O$	10.2 g
NaCl	2 g
$(NH_4)_2SO_4$	2 g
$MgSO_4$, $7H_2O$	2 g
$CaCl_2$, $2H_2O$	0.8 g
$ZnSO_4$, $7H_2O$	0.02 g
$FeSO_4$, $7H_2O$ (optional)	0.28 g

Dissolved in 1 l deionised water, pH 6.8, then autoclaved.

2.1.5.8. C+Y Medium

Combined the following sterile solutions to make semi-synthetic C+Y media.

PreC	400 ml
Phosphate Buffer 1M	15 ml
Supplement	13 ml
Glutamine (1 mg / ml)	10 ml
Adams III	10 ml
Yeast extract (5%)	9 ml
Pyruvate (2%)	5 ml
BSA (8%)	3.6 ml

Components of C+Y medium were made by the following recipe and mixed aseptically in the above mentioned proportions, filter sterilised and then stored in dark untill used.

PreC

Sodium acetate	1.2 g
Casamino acid (Difco, Vitamin free)	5 g
Tryptophan	5 mg
Cysteine	50 mg

Adjusted the pH to 7.5 by adding 10 N NaOH (~ 1 ml), and made up the volume to 1 l.

Autoclaved 200 ml portions in 500 ml flask.

Sugar solutions

20 % glucose and 50% sucrose sterilized by autoclaving.

2 % Pyruvate

2 g sodium pyruvate in 100 ml deionised water filter sterilized.

Glutamine (1mg / ml)

100 mg glutamine in 100 ml deionised water and filter sterilized.

Nucleoside solutions

2 mg / ml solution of adenosine and uridine, separately, autoclaved and then stored at room temperature for further use. Longer storage at lower temperature leads to precipitation.

3 in 1 salt

$MgCl_2$, $6H_2O$	10 g
$CaCl_2$, $2H_2O$	50 mg
$MnSO_4$, H_2O	4 mg

Dissolved in 100 ml deionised water and autoclaved.

Phosphate Buffer

1 M solutions of KH_2PO_4 and K_2HPO_4: Mix 26.5 ml of 1 M KH_2PO_4 with 473.5 ml of 1 M K_2HPO_4. There was no need to titrate the buffer. This gave pH 8.0 $\pm$ 0.2 to the final medium. Sterilized by passing through 0.45µ filter.

Adams I

Biotin	30 µg
Nicotinic acid	30 mg
Pyridoxine hydrochloride	35 mg
Pantothenate-Ca	120 mg
Thiamine hydrochloride	32 mg
Riboflavine	14 mg

Dissolved in 200 ml deionised water, filter sterilized and stored in dark at 4-8 ^oC.

Adams II

$FeSO_4$, $7H_2O$	50 mg
$CuSO_4$, $5H_2O$	50 mg
$ZnSO_4$, $7H_2O$	50 mg
$MnCl_2$, $4H_2O$	20 mg

Dissolved in 80 ml deionised water, then 1 ml concentrated HCl added. Made up the volume to 100 ml. Filter sterilized.

Adams III

Adams I	16 ml
Adams II	4 ml
Asparagine	200 mg
Choline chloride	20 mg
$CaCl_2$ (0.1 M)	160 µl

Dissolved in deionised water and made up the volume to 100 ml. Filter sterilized and stored in dark at 4-8 ^oC.

Supplement

Combined the following sterile solutions

3 in 1 salt	60 ml
Glucose 20 %	120 ml
Sucrose 50 %	6 ml
Adenosine 2 mg / ml	120
Uridine 2 mg / ml	120

BSA 8%

Dissolved 8 g BSA in 80 ml deionised water. To the stirring solution 10 N HCl is slowly added to bring down the pH to 1.5-2.5. Heat the solution in boiling water bath for 15 min, with occasional shaking. Leave it on table to cool down to room temperature. Neutralize it to pH 6-8 with NaOH. Make up the volume to 100 ml and take care that the BSA does not coagulate in any of the steps during preparation. Filter sterilize the solution and keep it at 4-8 ^{o}C in aliquots. This is an old Rockefeller recipe works very well as in pneumococcal growth media. Though simply dissolving 8 g BSA in deionised water also worked, it was preferred to follow this method while making media for growing competent cells for transformation.

2.1.5.9. Defined minimal medium for *Streptococcus pneumoniae* cultivation

Components	**Quantity per l**
Histidine	100 mg
Arginine	100 mg
Lysine	100 mg
Cysteine	100 mg
Methionine	50 mg
Asparagine	20 mg
Glutamine	20 mg
Threonine	20 mg
Isoleucine	10 mg
Leucine	10 mg
Valine	10 mg
NaCl	4 g
MOPS	3 g
NH_4Cl	2 g

K_2HPO_4	0.6 g
Pyruvate-Na	0.4 g
Glucose	2.5 g
Sucrose	0.6 g
BSA	0.8 g
$MgCl_2$, $6H_2O$	203 mg
$MnCl_2$, $4H_2O$	4 mg
$CaCl_2$, $2H_2O$	3 mg
$ZnCl_2$	30 µg
Na_2MoO_4, $2H_2O$	25 µg
Choline chloride	5 mg
Pantothenate-Ca	2 mg
Niacinamide	1 mg
Pyridoxal	1 mg
Thiamine hydrochloride	1 mg
Riboflavine	1 mg
Uracil	1 mg
Biotin	0.1 mg

2.1.6. Antibiotics

Amoxycillin, 10 mg / ml in 0.1 M phosphate buffer, pH 6.0.

Ampicillin, 10 mg / ml in 0.1 M phosphate buffer, pH 8.0.

Chloramphenicol, 10 mg / ml in 95% ethanol.

Erythromycin, 50 mg / ml and 1 mg / ml in 95% ethanol.

Nalidixic acid, 10 mg / ml in half volume water, then a minimum volume of 0.1 M NaOH was added to dissolve, then make up to total volume with water.

Kanamycin, 10 mg / ml in water.

Tetracycline, 10 mg / ml in 70% Ethanol.

Gentamycin, 10 mg / ml in water (for *in vitro* use) or endotoxin free 1x D-PBS (Gibco, Cat. No. 14040-091) for *in vivo* use.

Albomycin, 1mg / ml in water (for *in vitro* use) or endotoxin free 1x D-PBS (Gibco, Cat. No. 14040-091) for *in vivo* use.

Salmycin, 1mg / ml in water (for *in vitro* use).

2.1.7. Reagents and buffers

2.1.7.1. TAE Buffer (50x)

Tris-base	242 g
Glacial acetic acid	57.1 ml
EDTA 0.5 M (pH 8.0)	100 ml

Dissolved in deionised water and volume made up to 1 l.

2.1.7.2. DNA Loading Dye (10 x)

Bromophenol Blue	0.025 g
SDS 10%	1.25 ml
Glycerol	12.5 ml
Tris 10 mM	6.25 ml

2.1.7.3. SDS-PAGE of protein

Stocks

1. Acrylamide - Bis acrylamide mix (20 %, 30:1) add 20 ml acrylamide (30 % solution in water, Roth) to 10 ml bis-acrylamide (2 % solution in water, Roth)
2. 1 M Tris (pH 6.8) & 1 M Tris (pH 8.8)
3. SDS 10 %
4. APS 10 % & TEMED
5. Running buffer (1X & 5X)

Running gel (12 ml, 10 %), sufficient for 2 small gels or one big gel

Components	Quantity	Final concentration
Deionised water	1.6 ml	
Acrylamide mix 20%	6 ml	10 %
1 M Tris-HCl (pH 8.8)	3 ml	250 mM
SDS (10 %)	1.2 ml	1 %
APS (10 %)	200 µl	
TEMED	20 µl	

Stacking gel (6 ml, 5 %), sufficient for 2 small gels or one big gel

Components	Quantity	Final concentration
Deionised water	3 ml	
Acrylamide mix 20%	1.5 ml	5 %
Tris (pH 6.8, 1 M)	0.75 ml	125 mM
SDS (10 %)	0.6 ml	1 %
APS (10 %)	100 µl	
TEMED	20 µl	

4X PAGE Loading Buffer

To prepare 10 ml of 4X Gel Loading Buffer, dissolve the following reagents to 8 ml water.

Tris HCl	0.666 g
Tris Base	0.682 g
SDS	0.800 g
EDTA	0.006 g
Glycerol	4 ml
SERVA Blue G250 (1% solution)	0.75 ml
Phenol Red (1% solution)	0.25 ml

Mixed well and the volume adjusted to 10 ml with deionised water. Stored at +4°C.

Running buffer (Tris-Glycine buffer, 5X)

Components	Concentration	for 1 l
Tris-HCl	125 mM	15.1 g
Glycine	1.25 M	94 g
SDS	0.5 %	50 ml of 10% SDS

Bradford Reagent

Ethanol	5 ml
Orthophosphoric acid	10 ml
Coomassie Brilliant blue	10 mg

Coomassie Brilliant Blue was completely dissolved and adjusted to 100 ml with deionised water and filtered to remove blue particulates. This preparation (1 ml) gave linear curve within a range of 1-50 µg BSA.

Staining Solution

Coomassie Brilliant Blue (R 250)	0.25 g
Methanol	45 ml
Deionised water	45 ml
Glacial Acetic acid	10 ml

Destaining Solution

Methanol	40 ml
Deionised water	50 ml
Glacial Acetic acid	10 ml
APS (10 %)	200 µl
TEMED	20 µl

2.1.7.4. Protein purification buffers used

Lysis Buffer	20 mM Tris-HCl, 100 mM NaCl, 10% glycerol, pH 8.0 1 mM PMSF
Wash Buffer 1	250 mM NaCl, 50 mM Tris-HCl, 1 mM PMSF, pH 8.0.
Wash buffer 2	250 mM NaCl, 50 mM Tris-HCl, pH 8.0, up to 50 mM Imidazole, 1 mM PMSF
Elution buffer	50 mM Tris-HCl, 250 mM NaCl, pH 8.0, From 200 mM to 500 mM Imidazole

2.1.7.5. Binding protein buffer

Tris	20 mM
NaCl	100 mM
pH	7.4

2.2. Methods

2.2.1. Growth conditions

Escherichia coli and other collected strains of *Enterococcus faecium, Enterococcus faecalis, Staphylococcus epidermidis, Staphylococcus aureus*, *Listeria monocytogenes, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella heidelberg, Salmonella enteritidis, Serratia liquefaciens*, *Serratia marcescens, Klebsiella pneumoniae*, *Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa* were routinely grown in TY medium. The incubation temperature was 37 ^{o}C if not indicated otherwise. Liquid cultures were shaken in a rotary shaker at 200 rpm. *Yersinia enterocolitica* were grown, as *Escherichia coli* except the incubation temperature was 28 ^{o}C.

Streptococcus pneumoniae and other streptococci were grown in liquid cultures either in THY Broth or in C+Y medium without shaking at 37 ^{o}C in screw capped tubes. For growing different streptococci on solid medium, Mueller-Hinton Agar or THY agar supplemented with 5 % sheep blood were used. *Streptococcus* on plates were incubated at 37 ^{o}C with 5 % CO_2 and 95% relative humidity.

Streptomycetes were routinely grown on solid HA medium at 28 ^{o}C and for albomycin production in liquid albomycin production medium with shaking at 200 rpm at 37 ^{o}C. Incubation continued for 3-10 days before subculturing or for further experiments.

2.2.2. Fermentation of albomycin

Fermentation from *Streptomyces* ATCC 700974 for albomycin production was carried out in a 20 l fermenter at 28^{0} C for up to 138 h. In the fermenter 20 l of albomycin production medium without any extra-added iron was seeded with 20 ml of a 4-day-old culture of the selected strain carrying 1 mM iron leading to a 1 μM final iron concentration in the fermenter. Initial pH of the medium was 6.8, and dropped during the course of growth to near 6.0. Then NaOH was added to bring the pH up to 7. After 70 h ferrous sulphate was added to the growing culture to achieve a final iron concentration of 1 mM.

2.2.3. Chromatography

2.2.3.1. XAD-16 column chromatography

The column was loaded with Amberlite XAD-16 beads with a surface area 800 m^2/g, pore diameter ~ 250 A^0 , bed volume 1.5 l, column dimensions 8 cm x 30 cm, flow rate 7.5 l/h. The total culture filtrate was directly loaded on the column, the flow through discarded, washed with deionised water until a clear eluent was obtained. Bound fraction was eluted with 40% acetone (v/v) in 2.2 l volume. Columns were regenerated by passing through 100% methanol.

2.2.3.2. Gel exclusion chromatography of albomycin

Column of a dimension of 5 cm x 90 cm loaded with BioGel P2 (400 mesh) was used for gel exclusion chromatography. In 10 ml water XAD-16 lyophilised fractions were dissolved and loaded on the column and eluted with water. Fractions were monitored at 435 nm and later on by bioactivity test and by HPLC. Fractions of interest were pooled, evaporated and again lyophilised. The column was regenerated by passing 2 l of 0.4 % NaCl through water followed by 2 l of 0.4 % NaCl in 20% methanol.

2.2.3.3. Analytical and preparative HPLC of albomycin

Samples were analysed by analytical HPLC (Shimadzu LC10 pumps) on a reversed phase column (Nucleosil C18, 5 µM, 4 x 250 mm). Injection volumes of samples were 20 µl. Mobile phase was for gradient elution from 100% 2 mM ammonium acetate to 100% acetonitrile in 20 min with a flow rate of 2 ml / min. Standard albomycin δ_2 solution of 0.5 mg/ml was used as reference. The eluate was monitored at a wavelength of 220 nm. Determination limit was 1 µg / ml of albomycin. For purification of albomycin preparative HPLC was done with a Nucleosil C18, 7 µm column of dimension 20 x 250 mm. Sample injection volume was 1 ml, with the same mobile phase as in the analytical HPLC but with a flow rate of 5 ml / min for a total of 50 min.

2.2.4. Albomycin sensitivity assay

Sensitivity assays were performed with aqueous albomycin solutions. All strains that showed growth on TY plates were grown in 3 ml TY broth overnight. Separate TY agar plates were overlaid with 3 ml TY-soft agar thoroughly mixed with 20 µl of overnight grown cultures of test strains. TY plates overlaid with test organisms were directly spotted with 5 µl of the antibiotic solution and incubated at 37 ^{0}C for 16-18 hours. Inhibition zones indicated susceptibility and lack of inhibition resistance. Strains that showed apparent resistance on TY plates were also checked under iron-limited condition created with 200 µM dipyridyl. For this NBD plates were overlaid with water-dipyridyl-soft agar mixed with 20 µl of overnight grown cultures in TY broth. Albomycin was spotted on such plates. Results were noted after 16-18 h incubation at 37 ^{0}C. The laboratory strain *E. coli* SIP 401 was used as positive control for sensitivity to albomycin.

For testing *S. pneumoniae* cells were grown in THYB for 14-18 hours and then 50 µl of the culture was spread evenly with sterile cotton swab over blood agar plate. Filter paper discs containing the albomycin were placed on the plates. Results were checked 24 h after incubation at 37 ^{0}C.

2.2.5. Growth promotion assay

Under iron limited conditions siderophores (ferrichrome and ferrioxamines) at concentrations from 10 to 1000 µM were used to check growth promotion. For this NBD plates were overlaid with water-dipyridyl soft agar or with water-dipyridyl-EDDHA soft agar mixed with 20 µl of overnight grown cultures in TY broth. Aqueous solutions of ferrichrome or ferrioxamine (5 µl of 10, 100 and 1000 µM) were spotted on filter paper discs placed on such plates. Results were noted after 16-18 h incubation at 37 ^{0}C. As positive control for growth promotion with ferrioxamine B, *E. coli* MS 172 grown in NB was used and with ferrichrome *E. coli* AB 2847 was used.

2.2.6. Transformation in *E. coli*

2.2.6.1. Preparation of electrocompetent *E. coli* cells

1. Overnight culture in TY broth with vigorous shaking.
2. Culture 30 ml with 1% inoculum of the culture
3. Grow at 37 ^{o}C till OD578 reaches ~ 0.5 to 1.0 (0.7 optimum)
4. Spin 4000x g, 10 min, 4 ^{o}C
5. Wash twice with 10 ml cold sterile 10 % glycerol.
6. Resuspend in 600 µl MOPS-glycerol (1 mM MOPS, 15% glycerol)
7. Keep 50 µl aliquotes at -80 ^{o}C

2.2.6.2. Electroporation of *E. coli*

1. 50 µl cells + 4.5 µl DNA in an ice cold cuvette, on ice
2. Electroporate in a BioRad Genepulser under the following conditions

   25 µF

   100/200 Ω

   5 msec (optimum)

   2.5 kV/mm
3. Put back on ice for 30 sec
4. Add 2 ml recovery broth
5. Incubate at 37 ^{o}C with shaking for 1-2 h
6. Spread on selective medium

2.2.7. Transformation of *Streptococcus pneumoniae*

2.2.7.1. Preparation of competent *Streptococcus pneumoniae* cells

1. Start overnight culture of *S. pneumoniae* in C+Y medium at 37 ^{o}C.
2. Inoculate fresh C+Y medium + BSA with 1 / 100 volume of an 8-10 h preculture.
3. Incubate at 37 ^{o}C and follow the OD_{600}. When the OD_{600} reaches 0.2 add synthetic competent stimulating peptide to the culture to a final concentration of 200 ng / ml.
4. Keep at room temperature for 5 min (not longer).
5. Add sterile glycerol to a final concentration of 10 % (v/v).

6. Keep aliquots of 0.5 ml in -80 ^{o}C. Competence was assayed for one aliquot.
7. Frozen cells maintained competence for at least 3 months when multiple freeze-thaw cycles were avoided. Very good competence for one month.

2.2.7.2. Natural transformation in *Streptococcus pneumoniae*

1. Thaw frozen competent cells on ice.
2. Add transforming DNA (up to 1 µg) in a volume up to 10 µl in 2 ml sterile cup.
3. Add 100 µl of competent cells to the DNA sample.
4. Incubate at 30 ^{o}C for 40 min.
5. Add 1.5 ml THY broth (if not mentioned otherwise) and incubate at 37 ^{o}C for 2-3 h.
6. An appropriate amount of transformed culture was mixed with 10 ml melted THY agar and 0.5 ml sheep blood and poured on THY-Blood plates (15 ml) with 2X selective antibiotic.

Note While transforming chromosomal DNA, 50-250 µl of transformed culture was plated, and for insertion duplication mutagenesis with circular plasmid DNA, the whole transformed culture was plated.

2.2.8. Mutagenesis in *Streptococcus pneumoniae*

2.2.8.1. Insertion duplication mutagenesis (IDM) of target gene in *Streptococcus pneumoniae*

Loss of function mutants of targeted genes were regularly made by insertion duplication mutagenesis. Schematic Fig. 4 describes the principle of IDM as has been used for gene inactivation. Principally, internal 200-900 bp fragment of the target gene was cloned in plasmid pJDC9 that can replicate in *E. coli* but not in pneumococci. The recombinant plasmid with target insert was multiplied in *E. coli*, purified and transformed into pneumococci with wild type target gene. Single crossover between homologues sequence shared by introduced circular plasmid and genomic segment results in full insertion of plasmid sequence within the target gene. This essentially inactivates the target gene and the clones bearing the insertion can easily be selected with the help of the antibiotic resistance marker on the plasmid sequence.

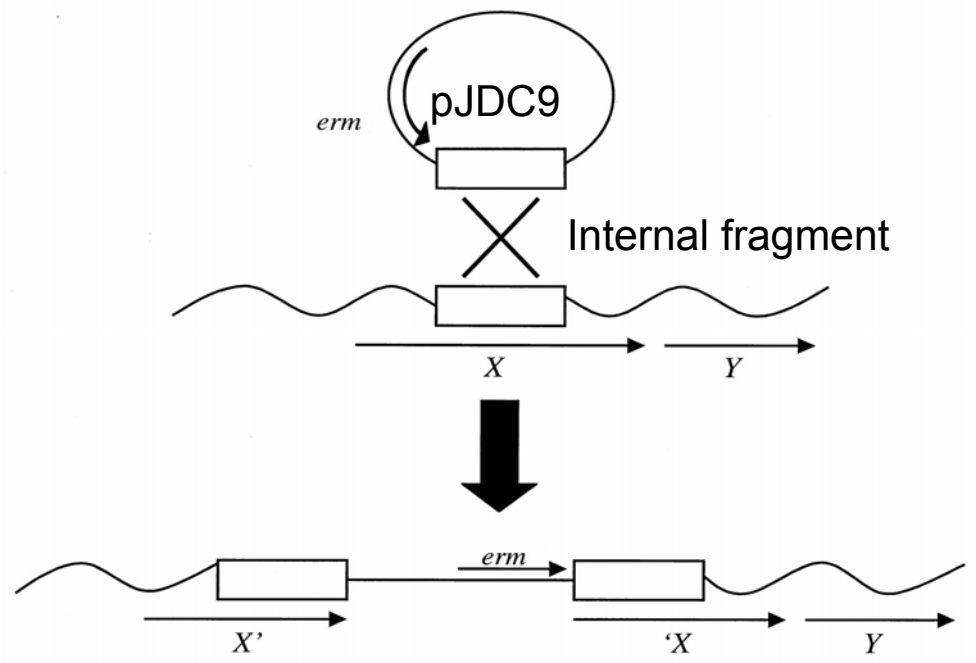


Fig. 4. Schematic presentation of the Insertion Duplication Mutagenesis (IDM) method performed in pneumococcus using pJDC9 plasmid.

2.2.8.2. Creating targeted deletion in the *Streptococcus pneumoniae* chromosome

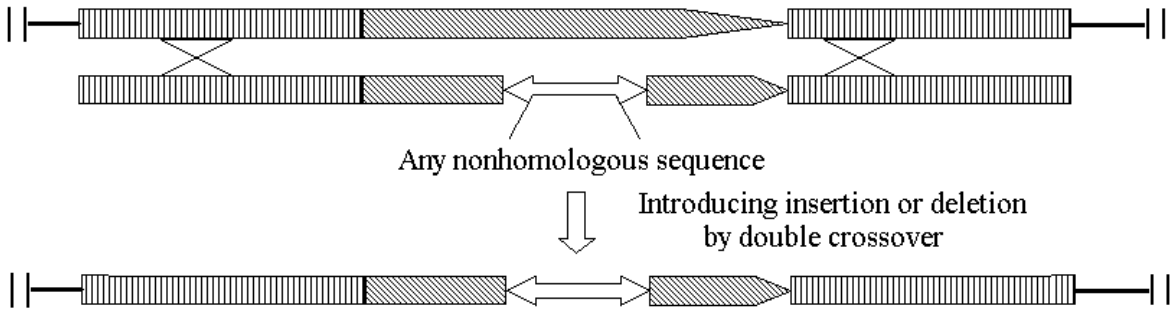


Fig. 5. Schematic presentation of the principle of homologous recombination been exploited in pneumococcus for making deletion mutant and transferring chromosomal insertions / mutations from one strain to another.

Given the unique ability of pneumococcus to take up linear naked DNA including PCR amplified products and to recombine them at homologous regions inside the chromosome enabled to design an approach to introduce *in vitro* created custom deletions in the chromosome. Principally, large chromosomal (~ 4 kb) fragment was PCR amplified,

necessary deletion was introduced in vitro following treatments of restriction endonucleases and ligase. The desired template was enriched and amplified by nested PCR, purified and transformed into pneumococcus with the expectation for a double crossover transferring the custom modified sequence in to the chromosome. Fig. 5 schematically presents the expected events of transformation to recombination. The same method can be used for creating either deletion or insertion or a combination of both, given the altered region is flanked by about 1 kb homologous sequence on either side.

2.2.8.3. *In vitro* random transposon mutagenesis in *Streptococcus pneumoniae*

EZ-Tn5™ transposase is a hyperactive form of Tn*5* transposase described by Goryshin and Reznikoff and made commercially available by Epicentre, USA (Goryshin *et al.*, 1998; Goryshin and Reznikoff, 1998; Goryshin *et al.*, 2000). This EZ-Tn5™ transposase can specifically and uniquely recognizes the outer end sequences of naturally occurring Tn*5* and mini-Tn*5* transposons and the hyperactive mosaic ends (ME) of EZ-Tn5™ transposons. Virtually the single-subunit transposes enzyme can be used to randomly insert any EZ-Tn5™ transposon into any target DNA *in vitro*.

A typical EZ-Tn5 transposition reaction requires four components (1) the EZ-Tn5 transposase; (2) an EZ-Tn5 transposon; (3) a target DNA; and (4) the presence of Mg^{2+}. The highly random insertion of an EZ-Tn5 transposon into the target DNA proceeds by a cut and paste mechanism, catalyzed by the EZ-Tn5 transposase, and results in a 9-bp duplication of target DNA sequence immediately adjacent to both ends of the transposon. Transposon has a specific 19 bp transposase recognition sequence (Mosaic End or ME sequence) at each of its ends. EZ-Tn5 transposase catalyzes a multi-step "cut and paste" transposition reaction. Initially, the enzyme binds the 19 bp ME of the transposon to form a Transposome™ (synaptic complex). The transposome then randomly attacks and cleaves the phosphodiester backbone of the target DNA. Finally, the EZ-Tn5 transposase catalyzes the covalent linkage of the 3'-OH ends of the transposon to the exposed 5-phosphorylated ends of the target DNA. Transposition creates a 9 bp sequence gap immediately flanking the transposon on either side of the insertion on different strands. For successful recombination by transforming into naturally competent *S. pneumoniae*, this gap filling would be necessary to allow introduction of heterologous sequence by double crossover event at homologous flanking regions (Akerley *et al.*, 1998; Hava and Camilli, 2002; Lau *et al.*, 2002; Majewski *et al.*, 2000; Mortier-Barriere *et al.*, 1997; Pasta and Sicard, 1999; Prudhomme *et al.*, 2002). The gap can not be

filled by the transposes alone. A set of enzymes involving DNA polymerase, polynucleotide kinase and ligase can carry out gap filling in the sequence, recovering the intact double strand after transposition.

2.2.8.4. In vitro transposon insertion reaction and transformable template preparation

This reaction inserts any EZ-Tn5 transposon into target DNA, *in vitro*.

1. Prepare the transposon insertion reaction mixture by adding in the following order

EZ-Tn5 10X Reaction Buffer	10 µl
Pneumococcal chromosomal DNA	70 µl
EZ-Tn5 transposon	18 µl
EZ-Tn5 transposase	2 µl
Total reaction volume	100 µl

2. Incubate the reaction mixture for 4 hours at 37 ^{o}C.
3. Stop the reaction by adding 10 µl EZ-Tn5 10X Stop Solution. Mix and heat for 10 minutes at 70 ^{o}C.
4. Add an equal volume of buffer-saturated phenolchloroform (150) to the DNA solution. Mix well gently.
5. Spin in a microfuge for 10 min.
6. Carefully remove the aqueous layer to a new tube, avoid the interface.
7. Repeat steps 4-6 with chloroform only to remove traces of phenol.
8. Remove aqueous layer to new tube.
9. Add 1/10 volume 3 M Na-Acetate and an equal volume of isopropanol.
10. Spin in a microfuge for 30 min at 14000 x g.
11. Wash the pellet with 70% ethanol.
12. Dry the pellet at room temperature, then dissolve in 33 µl of 5 mM Tris-HCl.
13. Add the following components to the tube with dissolved DNA

10X buffer for T4 polymerase or polynucleotide kinase	5 µl
2.5 mM dNTP Mix	5 µl
10 mM ATP	5 µl
T4 polymerase	1 µl
T4 polynucleotide kinase	1 µl
Total reaction volume	50 µl

13. Incubate at room temperature for 30 minutes.
14. Add 1 µl T4 DNA ligase (fast / quick) and 1 µl of 10 mM ATP to the reaction.
15. Incubate at room temperature for another 60-90 minutes.
16. Stop the reaction by heating at 70 ^{o}C for 15 minutes**.**
17. Use aliquot or the full reaction mixture to transform highly competent *Streptococcus pneumoniae.* Store unused reaction mixture at -70 ^{o}C.

2.2.9. Ferrichrome transport assays

The transport assays were carried out according to the published protocol (Hantke, 1983). Bacteria were grown on TY plate overnight. Cells were scraped off and suspended in prewarmed (at 37 ºC) M9 medium (M9 salts + 0.4% glucose). They were washed twice and finally diluted to OD_{578} = 0.5~0.6 with the same prewarmed medium. An aliquot of 0.7 ml of the diluted cell suspension was transferred to 2.0 ml cup and incubated at 37 ºC with shaking for 5 min after adding 17.5 µl of 10 mM nitrilotriacetate (NTA). At t=0 min 7 µl of radiolabelled ferrichrome mixture was added to the shaking suspension at 37 ºC. At different time points 100 µl of the suspension was withdrawn and filtered through nitrocellulose membrane filters of 25 mm diameter with pore size 0.45 µm. Cells remained on the membrane, and were washed twice with 5 ml of 0.1 M LiCl. Membranes were dried separately in prelabelled tubes at 80 ºC for 10 min. After bringing them to room temperature, 5 ml scintillation cocktail was added and kept at room temperature for 30-60 min. Radioactivity in each sample was then measured in a liquid scintillation counter and radioactivity recorded.

Radiolabelled ferrichrome mixture

Radioactive $FeCl_3$, 500 µM	20 µl
Deferri ferrichrome 10 mM	5 µl
HCl 0.2 M	5 µl
Deionised water	70 µl
Total volume	100 µl

Keep the mixture at room temperature for 30 min to allow Deferri ferrichrome to bind radioactive iron. Then use for the transport assay. All radioactive wastes were discarded in properly labelled bins.

2.2.10. Polymerase chain reaction (PCR)

The PCR was performed to amplify DNA fragments from either plasmid DNA or chromosomal DNA. The PCR reaction mixture (50 µl) was composed as follows

Components	Volume in µl	Final Concentration
H_2O	34	
5x HF Phusion Buffer	10	1x
10 mM dNTPs each	1	200 µM each
Forward Primer 10 µM	2	0.4 µM
Reverse Primer 10 µM	2	0.4 µM
Template	0.5	
Phusion DNA Polymerase 2 U/µl	0.5	

The PCR reaction was carried out in a RoboCycler®Gradient 40 Temperature Cycler (Stratagene) according to the following cycling parameters:

Cycle Description	Temperature in ^oC	Time	Number of cycles
Initial denaturation	98	1 min	1
Denaturation	98	10 sec	25-30
Annealing	52-64, depending on primer Tm	30 sec	
Extension	72	10 sec + 20 sec per Kb amplification product	
Final extension	72	7 min	1

The PCR fragments were usually run on 0.7 - 1% agarose gels in TAE, then stained with ethidium bromide and visualized under UV illumination.

2.2.11. DNA sequencing

DNA sequencing reactions from plasmid DNA, purified PCR products or from direct chromosomal DNA were carried out by Claudia Menzel with the auto sequencing kit (Pharmacia) at the Lehrstuhl Mikrobiologie/ Membranphysiologie, Universität Tübingen.

2.2.12. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation by SDS-polyacrylamide gel electrophoresis was carried out with the vertical gel electrophoresis apparaturs (Biometra). The protein samples were dissolved in 1x gel loading buffer and heated at 95 ºC for 5 min to denature the proteins. In each well of the gel 20 µl samples were loaded. The protein molecular weight standard (Fermentas) was used as marker to determine the size of proteins. The electrophoresis was run at 20 mA until the dye front was run to the bottom of the glass plates. Then the gel was removed from the apparatus and stained in Coomassie blue staining solution for 1 h and then destained in 10% acetic acid containing destaining solution.

2.2.13. Purification of His-tagged protein from *E. coli.*

2.2.13.1. Overexpression of recombinant protein

1. Grow the his-tag containing clone in 30 ml TY broth with appropriate antibiotic at 37°C overnight.
2. Inoculate 200 ml of TY media (with antibiotic) with 10 ml of the overnight cultures and grow at 37°C with vigorous shaking until an OD600 of 0.6 is reached (60–120 min).
3. Take a 0.5 ml sample immediately before induction.
This sample is the uninduced control, pellet cells and resuspend them in 50 µl of 4x SDS-PAGE loading buffer. Freeze and store the sample at –20°C until SDS-PAGE analysis.
4. Induce expression by adding IPTG to a final concentration of 0.8 mM.
5. Incubate the cultures for an additional 4 h. Collect a second 0.5 ml sample. This sample is the induced control; pellet cells and resuspend them in 50 µl 4x SDS-PAGE loading buffer. Freeze and store the sample at –20°C until SDS-PAGE analysis.
6. Harvest the cells by centrifugation at 4000 x *g* for 20 min.
7. Freeze and store cell pellet at –20°C until further use or proceed to purification process.

2.2.13.2. Purification of 6xHis-tagged proteins under native conditions

1. Thaw the cell pellet for 30 min on ice and resuspend the cells in 10 ml native Lysis Buffer with lysozyme.

2. Incubate on ice for 30-60 min. Mix 2–3 times by gently swirling the cell suspension. Sonicate intermittently to enhance lysis and to reduce the viscosity of the cell lysate.
3. Centrifuge lysate at 14000 x *g* for 30 min at 4°C to pellet the cellular debris. Retain the cell lysate supernatant. The supernatant contains the soluble fraction of the recombinant protein.
4. Add 5 µl 4x SDS-PAGE loading buffer to a 15 µl aliquot of the supernatant and store at –20°C for SDS-PAGE analysis.
5. Gently resuspend the Ni-NTA-Agarose (Qiagen) by inverting it several times.
6. Add 1 ml of resin to the supernatant and mix gently for 1 h.
7. Pass the mix through an empty column so that resin beads are retained.
8. Collect the flow-through fraction. Add 5 µl 4 x SDS-PAGE loading buffer to a 15 µl aliquot of the flow-through fraction and store at –20°C for SDS-PAGE analysis.
9. Wash the column 3 times with 10 ml of native Wash Buffer. Collect all wash fractions. Add 5 µl 4x SDS-PAGE loading buffer to a 15 µl aliquot of each wash fraction and store at –20°C for SDS-PAGE analysis.
10. Elute bound 6xHis-tagged protein with four 1 ml aliquots of Native Elution Buffer.
11. Collect each elution fraction in a separate tube. Add 5 µl 4x SDS-PAGE loading buffer to a 15 µl aliquot of each elution fraction and store at –20°C for SDS-PAGE analysis.
12. Analyze all fractions by SDS-PAGE.

2.2.14. Determination of protein concentration

Prepared Bradford Reagent (1 ml) gives a linear curve within a range of 1-50 µg BSA. Samples were mixed with 1 ml of the reagent, vortexed vigorously, kept at room temperature for 15 min and then OD was taken at 590 nm wavelength.

2.2.15. Nucleic acid extraction and purification

2.2.15.1. Isolation of genomic DNA from bacteria

Stocks Macherey-Nagel Nucleobond buffers (G3, G4, N2), proteinase K, RNase A and lysozyme. All except buffer N2 were stored at 4 °C.
1. Dissolve RNase A in buffer G3 (final concentration 200 µg/ml). Dissolve proteinase K in autoclaved deionised water (20 mg/ml). Store buffer G3 and the proteinase K solution at 4 °C. If lysozyme is required, dissolve it in H_2O (100 mg/ml).

2. Pellet the bacterial cells from 3-10 ml culture by centrifugation at 4000 x g for 10 min. Discard the supernatant.
3. Resuspend the bacterial pellet in 500 µl buffer G3 by vortexing.
4. Add the lysozyme (optional) and the proteinase K stock solution, 10 µl of lysozyme and 10 µl of proteinase K. Incubate the mixture at 37 °C for 30 min. For *Streptococcus pneumoniae* add 2 µl of 2 % Na-deoxycholate instead of lysozyme.
5. Add 200 µl of buffer G4 and mix by vortexing.
6. Incubate the mixture at 60 °C for 20 min. If the lysate is not clear after incubation with proteinase K the incubation time can be prolonged.
7. Add buffer 500 µl of buffer N2 (room temperature) to the sample and mix by vortexing.
8. Add 500 µl of phenol-chloroform (11) and mix by vortexing.
9. Spin at 12000 x g for 20 min at 4 °C.
10. Aspire the aqueous phase and add equal volume of chloroform-isoamyl alcohol (24 1).
11. Mix by vortexing and spin at 12000 x g for 20 min at 4 °C.
12. Repeat from step 10.
13. Aspire the aqueous phase and add equal volume of chilled isopropanol. Mix by inverting the tubes few times.
14. Spin at 12000 x g for 30 min at 4 °C.
15. Discard the supernatant and wash the pellet with 70% ethanol.
16. Dry the pellet in air and dissolve in 5 mM Tris-HCl pH 8.0.
17. Check the quality and quantity by running an agarose gel.

2.2.15.2. Extraction of plasmid DNA from *E. coli*

1. Pick a single colony from a fresh plate and use it to inoculate 3–6 ml of LB plus an appropriate antibiotic. Incubate the culture overnight at 37 °C with shaking.
2. Harvest the fully grown culture in a 2 ml microcentrifuge tube and centrifuge at 10000 x g for 60 s at 4°C to pellet the bacterial cells. Discard the supernatant.
3. Carefully resuspend the pellet in 500 µl of Buffer A1+ RNAse.
4. Add 500 µl of Buffer A2 to the suspension. Mix gently by inverting the tube 6–8 times, and incubate at room temperature for 5 min (not more).
5. Add 600 µl of Buffer A3 to the suspension. Mix gently by inverting the tube.
6. Keep on ice for 10 min, then spin the suspension at 12000 x g in a microcentrifuge for 10 min at 4°C.

7. Aspire 1 ml of the clear supernatant without any debris in a fresh 2 ml microcentrifuge tube.
8. Add 800 µl of chilled isopropanol to the supernatant, mix it by brief vortexing.
9. Spin at 12000 x g for 30 min at 4 °C.
10. Discard the supernatant and wash the pellet with 70% ethanol.
11. Dry the pellet in air and dissolve in 5 mM Tris-HCl pH 8.0.
12. Check the quality and quantity by running an agarose gel.

2.2.15.3. Nucleic acid purification from agarose gels or PCR reaction mixtures using the NucleoSpin Extract kit

1. Excise gel slice containing the DNA fragment carefully with clean scalpel to minimize the gel volume. Transfer the gel slice to a preweighed clean tube and measure the weight. Determine the weight of the gel slice by subtracting both weights.
2. For each 100 mg agarose gel add 300 µl buffer NT1. For every 100 µl PCR product add 300 µl of Buffer NT2.
3. Incubate sample at 50°C until the gel slices are dissolved. Vortex the sample briefly every 5 min until the gel slices are dissolved completely.
4. Place a NucleoSpin Extract column into a 2 ml collecting tube and load the mixture.
5. Centrifuge for 1 min at 10000 x g. Discard flow-through and place the NucleoSpin Extract column back into the collecting tube. Repeat the step if necessary.
6. Add 600 µl buffer NT3 to the column. Centrifuge for 1 min at 12000 x g. Discard flow-through and place the NucleoSpin Extract column back into the collecting tube. Repeat this step once more.
7. Place the column into a fresh dry 1.5 ml microcentrifuge tube and centrifuge for 2 min at 12000 x g.
8. Place the NucleoSpin Extract column into a clean 1.5 ml microcentrifuge tube. Add 50 µl 5 mM Tris-HCl pH 8.0. Incubate at room temperature for 5 min to increase the yield of eluted DNA.
9. Centrifuge for 2 min at 12000 x g.
10. Check the quality and quantity by running an agarose gel.

2.2.16. Proteolytic digestion of binding protein

1. Dialyse his-tagged substrate binding protein against binding protein buffer overnight at 4 °C.
2. Take 20 µg of binding protein in binding protein buffer and incubate for 15 min at room temperature with or without 1 µM of test substrate.
3. Add 4 µg of either proteinase K and incubate for 30 min at room temperature.
4. Stop digestion by adding 1 mM PMSF (final concentration).
5. To precipitate whole protein from the reaction mixture, add an equal volume of 20% TCA (trichloroacetic acid), mix by repeated inversion of the tubes.
6. Keep on ice for 30 min, then centrifuge in microfuge at 4 °C for 15 min.
7. Wash the pellet with acetone.
8. Dry the pellet in air and dissolve it in 2x PAGE loading buffer.
9. Run SDS-PAGE to analyse degree of digestion.

2.2.17. Presumptive Tests for pneumococcus identification

2.2.17.1. Optochin susceptibility test

Ethyl hydrocupreine hydrochloride (Optochin) is a quinine derivative that is used to differentiate pneumococci from other viridans streptococci, with a sensitivity of greater than 95%, because of its ability to selectively inhibit the growth of *S. pneumoniae* on blood agar plates at very low concentrations (≤5µg/mL). The Optochin test is performed on a blood-agar medium using a disk diffusion principle. A few well-isolated colonies of the organism in question are streaked onto a blood-agar plate and a filter paper disk, impregnated with optochin, is placed in the streaked area. The plate is incubated and examined after 18 to 24 hours. Pneumococci surrounding the disk are lysed forming an inhibition zone of 14 mm or more around a 6-mm disk. If the inhibition zone is less than 14 mm, further testing (bile solubility or serology) is indicated.

2.2.17.2. Bile solubility test

The addition of bile salts, such as sodium deoxycholate, accelerates natural lysis of pneumococcal cultures by increasing the activation of autolytic enzymes produced by *S. pneumoniae*. The bile solubility test is performed by adding a bile-salt solution to an established broth or blood-agar culture of the organism in question. A positive result in broth

culture is obtained by noting visible clearing of the culture's turbidity, as compared to a control tube, after addition of the bile salt solution and re-incubation for up to 30 min. On blood-agar plates, bile-soluble pneumococcal colonies "disappear" leaving behind their green zone of α-haemolysis, after placing a drop or two of the bile-salt solution on the colony and re-incubating the plate for 30 minutes.

2.2.18. In vivo infection model

All in vivo mice work was done following university regulation in bio safety level containment 2 under specific pathogen free facility provided by Medical Clinic, Tuebingen, Germany and Dept. of Microbiology, University of Adelaide, Australia.

2.2.18.1. *Yersinia enterocolitica* infection model

Mice (C57 BL6) were bought from Harlan Gmbh., Germany at 4-6 weeks of age and acclimatized under specific pathogen free laboratory condition for 1-2 weeks. *Y. enterocolitica* 8081 strain were passed through mice to enrich mice virulent variants. Colonies recovered from mice were enriched once more by again passing through mice. Severely ill mice were sacrificed and bacteria were recovered and grown in TY broth to log phase and kept as glycerol stock until used. Infection inoculum was prepared by washing the bacteria in glycerol stock three times with PBS. To each mouse 5 x 10^4 bacteria suspended in PBS were injected intravenously in a volume of 200 µl. With this infection dose reliable bacterial count in spleen could be recorded within 24 h post infection. To obtain bacterial counts from infected spleen, infected mice were sacrificed at defined time points, spleen was removed aseptically and quickly immersed in 5 ml of cold PBT (PBS with 1% tergitol). Spleen was homogenized in that PBT and serial dilutions were plated on Mueller-Hinton agar plates. CFU was recorded 40 h after incubation at 28 ^{0}C. Antibiotics were injected intravenously as per scheduled time and dose in 100-200 µl volumes.

2.2.18.2. *S. pneumoniae* infection model

The BALBc mice were obtained from the Waite Agricultural Institute, University of Adelaide. Six mice per group were infected with 0.1 ml serum broth containing approximately 5×10^2 CFU of *S. pneumoniae* by intraperitoneal injection. Mice were subsequently treated by intraperitoneal injection of albomycin (at 10 mg/kg, 5 mg/kg or 1

mg/kg body weight), amoxicillin (10 mg/kg body weight) or PBS (control) at the time points described. Mice were bleed from the tail vein at various time point (Stroeher *et al.*, 2003). Blood (approx. 15 µl) was obtained using heparinized capillary tubing. The blood was serially diluted in PBS and plated onto blood agar with or without selection to recover viable *S. pneumoniae*. The competitive disease model is essentially as described above except that mice were infected by intraperitoneal injection with a mixture of the virulent wild type D39 parent and the albomycin resistant mutant at a ratio of approximately 100:1. In this case, the blood was serially diluted and plated onto blood agar or blood agar supplemented with erythromycin to determine the numbers of wild type and mutant *S. pneumoniae*.

3. Results

3.1. Fermentation of albomycin and purification to homogeneity

Albomycin is a naturally occurring sideromycin. The siderophore moiety of albomycin δ_2 is similar to ferrichrome. It contains three molecules of δ-N-hydroxy-δ-N-acetyl ornithine linked to a serine, all by peptide linkage. The C-terminus of the serine is linked to another serine attached to the antibiotically active 4'-thio (N^4-carbamoyl-3-methyl) cytidine moiety (Hartmann *et al.*, 1979). The trihydroxamate part serves the siderophore function; it can trap Fe^{+3} and is essential for active transport of the antibiotic. Antibiotically active free thioribosyl antibiotic moiety inhibits seryl tRNA synthetase (Stefanska *et al.*, 2000). Iron-free albomycin δ_2 has a molecular weight of 992 Da, and when loaded with iron it is 1045 Da.

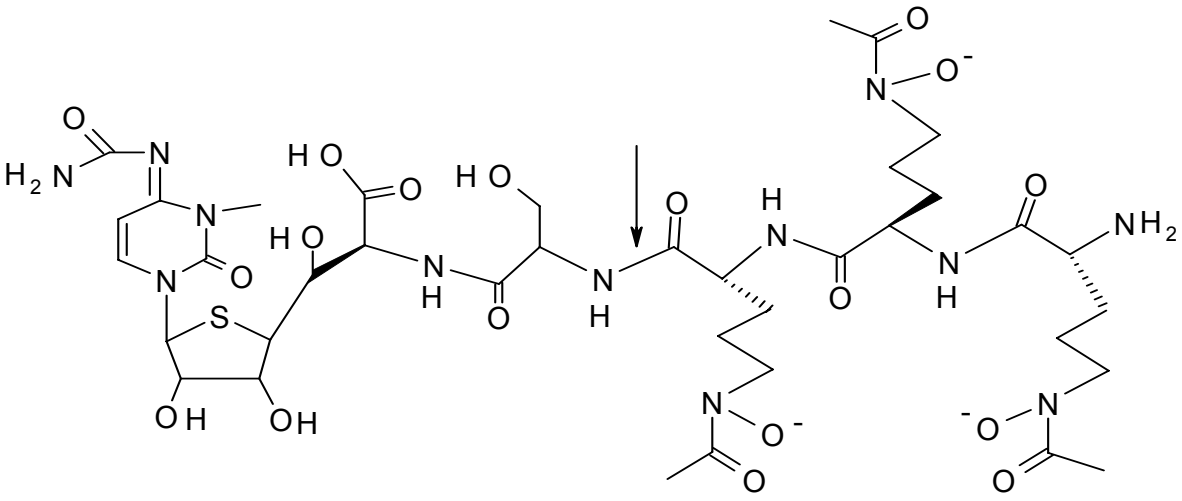


Fig 6. Chemical nature of albomycin δ_2, (↓) indicates peptidase N cutting site to release the antibiotic moiety from the trihydroxamate part.

For the present work albomycin production was standardized and then purified from a collection of albomycin producing *Streptomyces* strains.

3.1.1. Clone selection

Four albomycin producing *Streptomyces* strains from the culture collection were grown separately on HA plates for five-six days to produce spores. Spores from each plate (representing each strain) were scraped with a loop and collected in sterile water. The

suspensions were passed through a cotton pad sitting on a sterile 5 ml syringe to get rid of mycelia fractions. Resultant spore suspensions were centrifuged at 4000 rpm for 10 min, the supernatants discarded and the spore pellets resuspended in 1 ml sterile water. HA plates were streaked with this suspension and the rest was stored as glycerol stocks. Streaked plates were incubated at 28^0 C for 4 days. Individual colonies (24 from each strain) were spotted on albomycin production medium and incubated at 28^0 C. After 4 days these plates were overlaid with water-soft agar carrying 50 µl of an overnight grown *E. coli* SIP401 culture. After overnight incubation at 37^0 C, plates were checked for inhibition zones, which were supposed to be formed by albomycin. Colonies showing growth inhibition of the test strain were picked to inoculate 10 ml of albomycin production medium. On the 3rd and 4th day of incubation at 28^0C culture supernatants (after centrifugation at 10000 rpm, 5 min) from each flasks were checked for bioactivity on the same test strain. Among the four different *Streptomyces* strains tested for albomycin production, culture supernatant from two individual clones of ATCC 700974 strain showed best visible inhibition zone when applied to a lawn of *E. coli* SIP401. Both were pooled and allowed to form spores. Further selection for albomycin production from the lineage of individual spores yielded the best albomycin producing clone. This clone was maintained by continuous sub-culturing avoiding spore formation, and used for further standardization of albomycin production medium.

3.1.2. Optimisation of fermentation medium

Optimal fermentation condition for high albomycin production had been previously studied for *Streptomyces griseus* Tü 6 (Fiedler, 1985). Minor variations to this albomycin production medium have been tried to culture the selected clone along with the unmodified control, and albomycin yield was compared. For all the experiments the inoculum's size was 10^{-3} times of the culture volume. Without external adjustment the pH of the albomycin production medium was 6.8, suitable for growing the selected clone. Change of the carbon source from starch to maltose and glucose reduced the albomycin yield. One of the components of albomycin is ornithine, and addition of ornithine to the medium was found to be beneficial. The highest yield of albomycin obtained with 100 mM ornithine and 70 mM PO_4. Na_2HPO_4 was added after solubilizing all other components of the medium as it interferes with the solubility of the other components. Addition of Na_2HPO_4 causes precipitations in the medium, which dissolve in the course of cultivation. Triggering of production of albomycin may be attributed to the initial limited bioavailability of hard metal

ions caused by phosphate precipitation in the medium. The unmodified albomycin production medium contained 1 mM iron. The basic albomycin production medium was supplemented with varying concentration of iron and ornithine and albomycin production was followed. The initial iron concentration of the medium was varied from 1 μM to 1 mM. All media were supplemented with iron to a final 1 mM concentration after 3rd day of cultivation reaching the stationary phase. The otherwise yellowish medium promptly turned reddish with the addition of iron, indicating the presence of siderophores and related molecular species. Culture supernatants were checked at 30 min intervals for 3 hours after adding iron but they showed no bioactivity. The next day after iron supplementation they showed bioactivity, which was maintained at that level for 3 days which was not the case with the unmodified medium, where bioactivity started from 2nd day and reached the highest values on 3rd and 4th day, after which it declined. Following table shows the antibacterial activity of culture filtrates

Table 5. Albomycin quantification in culture supernatant of ATCC 700974 grown with varying iron and ornithine supplement.

Albomycin production medium			Inhibition zone diameter in mm after day				End point inhibition dilution of the highest production
Initial ornithine	Initial iron	Supplemented iron	3rd	4th	5th	6th	
30 mM	1 mM	-	**18**	17	16	12	300
30 mM	0.1 mM	1 mM	**17+**	16	14	8	200
30 mM	0.01 mM	1 mM	**16**	13	11	7	100
30 mM	0.001 mM	1 mM	-	16	**19**	18+	500
100 mM	1 mM	-	**19**	19	18	17+	500
100 mM	0.001 mM	1 mM	-	19	**20**	19+	700

3.1.3. Growth of the selected clone

The growth curve was measured from a 100 ml culture. At each time point 50 μl cultures was taken and total protein content determined with the Bradford Reagent.

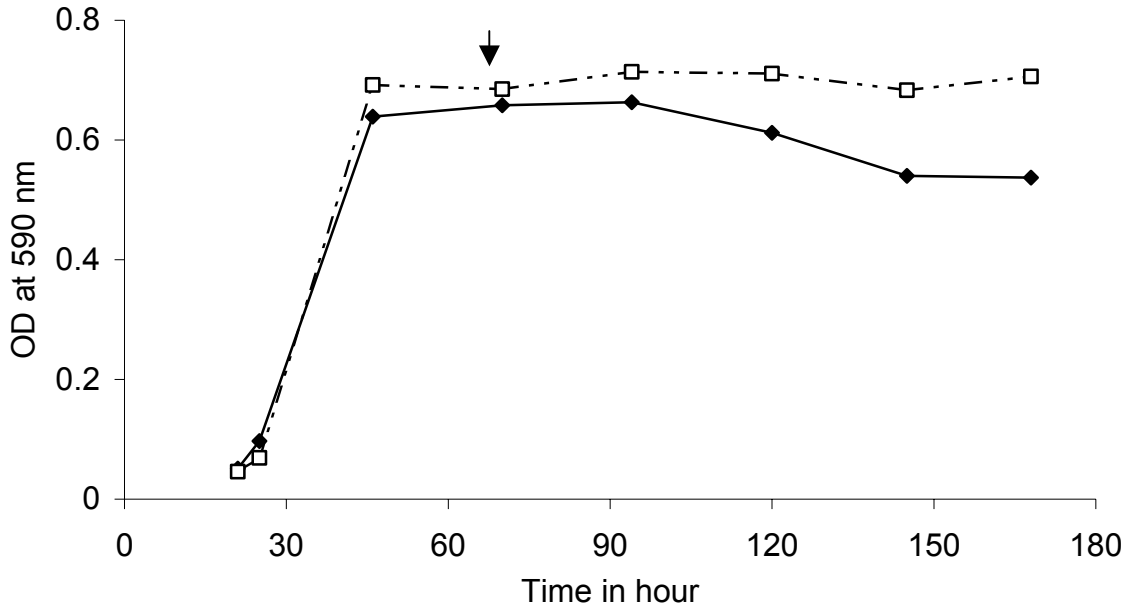


Figure 7. Growth curve of ATCC 700974 in albomycin production medium with 1 mM(——) and without (----) initially added iron, which was supplemented with 1 mM iron at after 70 h indicated by (↓).

Finally fermentation was carried out in the albomycin production medium with 1 µM iron (initially), supplemented with 1 mM iron in the form of ferrous sulphate 70 h after adding the inoculum. Fermenter (20 l) was seeded with 20 ml of the selected clone grown in albomycin production medium for 4 days. Cells were harvested after 138 h.

3.1.4. Purification of albomycin δ_2

From the 20 l fermenter 16 l of culture was harvested, the discrepancy of the volume could be attributed to loss through evaporation and removing samples. Chitan (2%) was added to this culture to improve filtration of the cells. A total of 14 l of culture filtrate was obtained, which was passed through a XAD-16 column (bed volume 1.5 l) with a flow rate of 7.5 l/h. The column was thoroughly washed with deionised water with the same flow rate until the eluent was clear. Adsorbed organic molecules were eluted from the column with 40% (v/v) acetone in water. Reddish-brown fractions (2.2 l in total) were collected and evaporated to a volume of 350 ml in a rotary evaporator, and then lyophilised for 3 days to obtain 4.3 g dry product. From this, 2.5 g were dissolved in 10 ml water and loaded onto a Bio Gel P2 column (5 cm x 90 cm). Fractions were eluted with water with a flow rate of 100 ml/h.

Fractions were monitored at 435 nm. Two major peaks were obtained, the largest one from fraction no. 33-38 and another from fraction no. 40-50. All the fractions were tested for bioactivity. All the fractions from 19 to 70 showed some bioactivity, with the bioactivity peak at fraction no. 40-50. Both peak fractions were pooled, evaporated separately to about 20 ml and then lyophilized. Fractions 33-38 yielded 0.3 g and the fractions 40-50 yielded 0.5 g of lyophilisate. Both samples were taken to make a 1 mg/ml solution that was then used for HPLC quantification along with 0.5 mg/ml reference solutions of albomycin δ_2. On bioactivity analysis, fraction 33-38 was found to contain ferrioxamine/s, which was confirmed by HPLC as ferrioxamine D1 and ferrioxamine E. Similar procedure was applied to the rest 1.8 g XAD-16 lyophilisate. All the eluted fractions were checked for bioactivity. From these results fraction 35 to 39 were pooled, evaporated and then lyophilized to 0.15 g. Lyophilized pooled BioGel fractions were quantified by HPLC and showed purity of albomycin δ_2 of about 25% in the 1st BioGel lyophilisate and 40% in 2nd BioGel lyophilisate. Concentration of albomycin determined by HPLC was less than that measured by comparing the bioactivity with the reference solution. According to HPLC quantization the recovered albomycin was 185 mg (= (500 x 0.25) + (150 x 0.4)).

Table 6. Quantification of albomycin recovery at different steps of purification.

	Inhibition zone diameter (mm)	End point dilution	Estimated concentration (µg/ml)
Reference Albomycin (1 mg/ml)	27	10000	1000
Reference Albomycin (10 µg/ml)	15	100	10
Culture filtrate, 14 l	18+	400	40
XAD-16 elute lyophilized, 4.3 g, 1 mg/ml	22	1200	120
1st BioGel fraction 40-50 lyophilized, 0.5 g, 1 mg/ml	25	4000	400 (250)
2nd BioGel fraction 35-39 lyophilized, 0.15 g, 1 mg/ml	26	7000	700 (400)

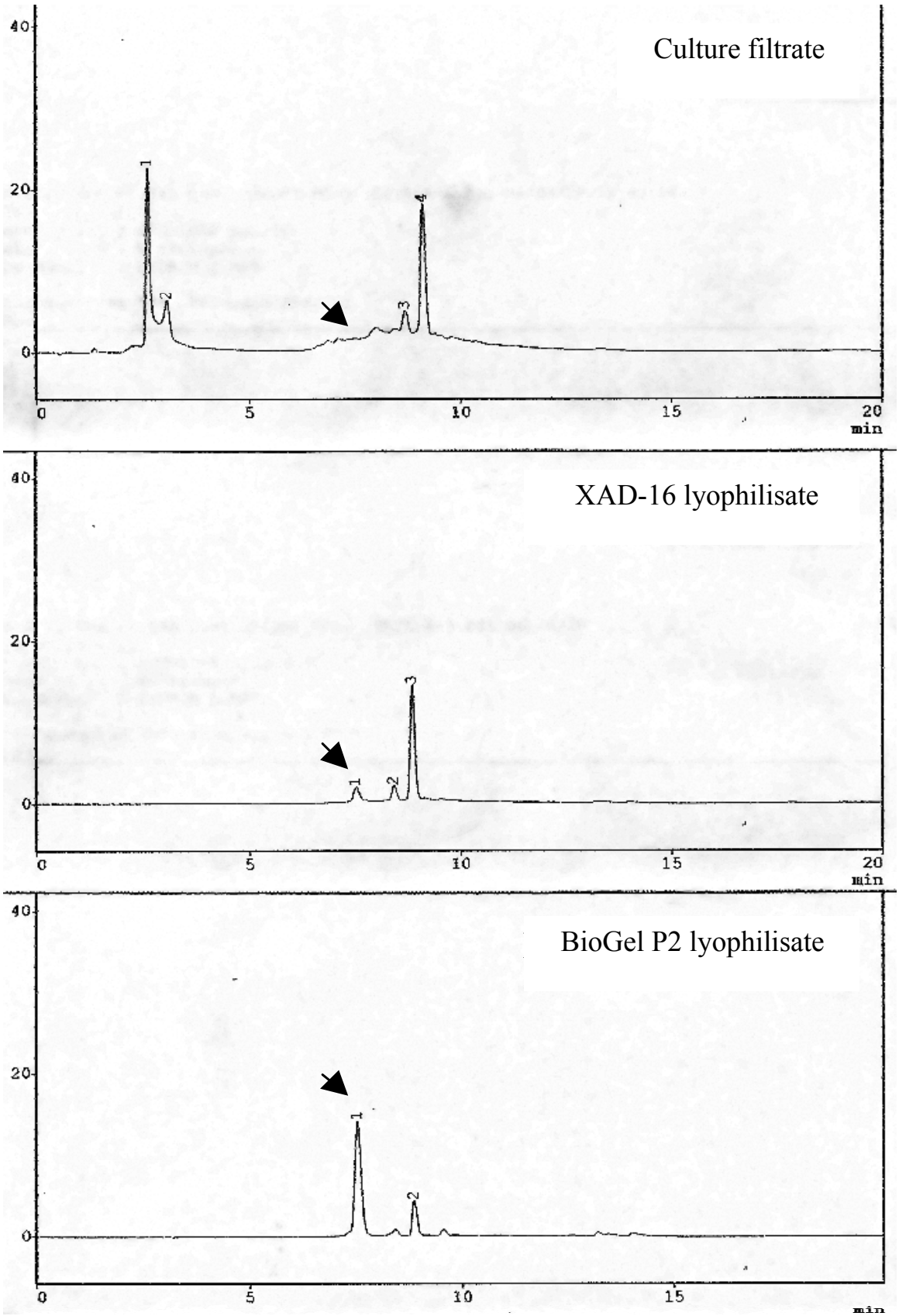


Fig 8. Sequential enrichment of albomycin at different steps of purification. Analytical HPLC at different steps of purification monitored at 435 nm to observe enrichment of albomycin δ_2. Albomycin δ_2 peak is marked by an arrow.

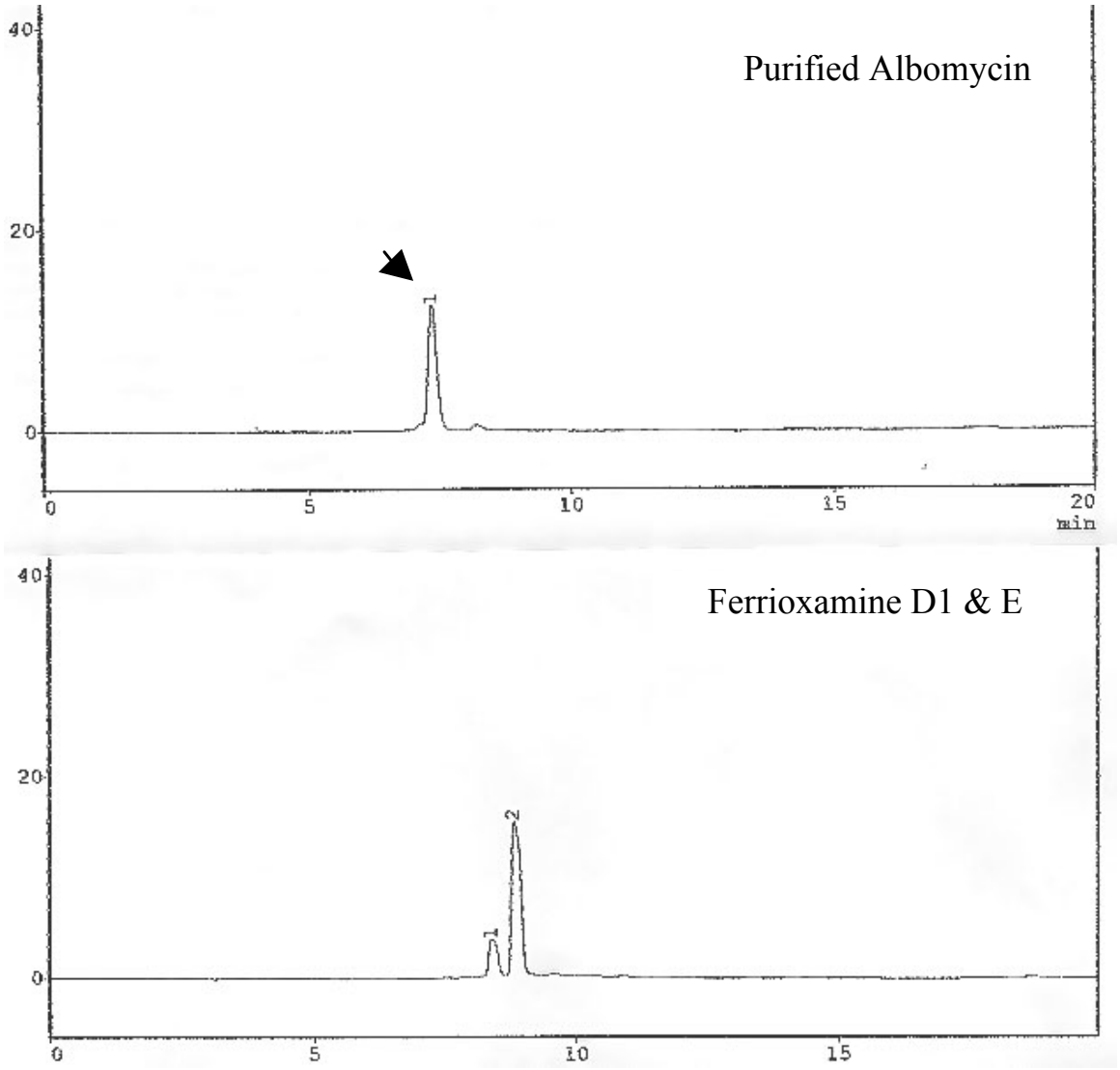


Fig 9. Analytical HPLC of purified albomycin δ_2 monitored at 435 nm (upper panel). Albomycin δ_2 peak is marked by arrow. Other BioGel fraction showing siderophore activity was found to be a mixture of ferrioxamine D1 and ferrioxamine E (lower panel).

In vitro experiments were initially done with BioGel purified albomycin preparation. Later for *in vivo* experiments lyophilised BioGel fractions were purified through preparative HPLC to obtain purest albomycin δ_2 (~100%). This preparation had no conceivable ill effect when injected via intra-venous or intra-peritoneal route in mice up to a dose of 10 mg / kg per injection thrice a day.

3.2. Activity spectrum of albomycin against pathogenic bacterial species

In a t-RNA synthetase inhibition assay used to isolate new antibiotics, the seryl-thioribosyl pyrimidine moiety of albomycin, designated SB-217452, was isolated from the culture supernatant of *Streptomyces* species ATCC 700974 and shown to inhibit *in vitro* seryl-t-RNA synthetase. SB-217452 is highly active against isolated t-RNA synthetase of *S. aureus*, (IC_{50} value of 8 nM), but poorly active against bacteria, e.g. MIC of 256 μg/ml (0.4 mM) for whole cell *Staphylococcus aureus*, presumably because of its low permeation into the bacterial cells (Stefanska *et al.*, 2000). Albomycin being a hydrophilic molecule with a molecular weight of about 1045 Da, cannot pass through porins. In contrast, complete albomycin is actively transported in energy-coupled steps across the outer and the cytoplasmic membranes of *E. coli* (Stefanska *et al.*, 2000). In *E. coli* albomycin crosses the membrane barriers via the Fhu-transport system, the same system used to transport ferrichrome. Most antibiotics enter cells by diffusion. The results obtained with *E. coli* and albomycin demonstrate that coupling of antibiotics with low permeation rates to actively transported molecules strongly increases the efficacy of the antibiotics (Braun *et al.*, 1983; Braun *et al.*, 2001; Ferguson *et al.*, 2001; Fischer *et al.*, 1989; Hartmann *et al.*, 1979; Koster and Braun, 1990; Rohrbach *et al.*, 1995). Deferri-ferrichrome is synthesized by fungi, secreted, complexes Fe^{3+} in the medium with an extremely high specificity and affinity, and is then transported into the producing fungi and certain bacteria by ferrichrome-specific transport systems(Ecker *et al.*, 1982; Emery, 1966; Ong and Emery, 1972; Schwecke *et al.*, 2006; Siegmund *et al.*, 1991). Many pathogenic bacteria were reported to posses hydroxamate specific transport systems (Autenrieth *et al.*, 1991; Baumler and Hantke, 1992; Biosca *et al.*, 1996; Jin *et al.*, 2006; Kornreich-Leshem *et al.*, 2005; Mikael *et al.*, 2002). To get a broader view of the distribution and dispensability of hydroxamate siderophores transport systems among pathogenic bacterial species the activity of albomycin was tested among fresh clinical strains along with laboratory strains.

3.2.1. Activity against *E. coli*

Albomycin sensitivity of clinical *E. coli* strains were determined and compared with the hypersensitive iron-deregulated laboratory strain SIP 401 *fur*. The sensitivity to albomycin varied widely from strain to strain and only 3 out of 23 strains tested were resistant (Table 7).

Table 7. Albomycin (2 mg / ml BioGel preparation) sensitivity of various clinical *E. coli* isolates with reference to the hypersensitive strain SIP 401.

Strain designation	**Inhibition Zone (mm)**
Escherichia coli SIP 401 (*fur*$^-$)	31
1. *Escherichia coli* 294	18
2. *Escherichia coli* 295.1	25
3. *Escherichia coli* 429	25
4. *Escherichia coli* 430	23
5. *Escherichia coli* 537.2	26
6. *Escherichia coli* 563.2	-
7. *Escherichia coli* 605.2	26
8. *Escherichia coli* 608.1	26
9. *Escherichia coli* 618.2	25
10. *Escherichia coli* 628.1	25
11. *Escherichia coli* 628.2	-
12. *Escherichia coli* 633.2	23
13. *Escherichia coli* 634.1	25
14. *Escherichia coli* 636.2	26
15. *Escherichia coli* 637.3	27
16. *Escherichia coli* 643.1	21
17. *Escherichia coli* 643.2	19
18. *Escherichia coli* 643.3	21
19. *Escherichia coli* 650.1	-
20. *Escherichia coli* 100537.1	22
21. *Escherichia coli* 100560.1	25
22. *Escherichia coli* 100564.2	24
23. Escherichia coli 100614.1	24

These three resistant strains showed reduced growth in TY and NB medium, in comparison to other strains. They were inoculated in 3 ml NB and OD was taken after 6 h incubation at 37 ^{0}C.

Table 8. Growth comparison of albomycin sensitive and resistant *E. coli* isolates in TY medium.

Strain designation	**Inhibition Zone (mm)**	**OD at 578 nm**
Escherichia coli 563.2	-	0.169
Escherichia coli 628.2	-	0.176
Escherichia coli 650.1	-	0.232
Escherichia coli 643.2	19	0.402
Escherichia coli 637.3	27	0.484

3.2.2. Albomycin sensitivity of common pathogens

Albomycin sensitivity of a wider array of gram positive and negative pathogenic bacteria is summarised in Table 9. Most *Enterobacteriaceae* species were found to be sensitive except *Proteus* and *Morganella*. Albomycin was ineffective against *Pseudomonas* and related *Burkholderia* and *Stenotrophomonas*. Whereas *Alcaligenes xylosoxidans*, another species frequently found in cystic fibrosis patients along with pseudomonads, found to be sensitive to albomycin when grown under iron-limited conditions. *Haemophilus - Pasteurella - Mannheimia* group were albomycin resistant.

Table 9. Albomycin sensitivity of clinical isolates of gram-negative bacterial pathogens.

Pathogenic strains (number tested)	Albomycin sensitivity
1. *Escherichia coli* (24)	+
2. *Serratia marcescens* (3)	+
3. *Serratia liquefaciens* (3)	+
4. *Klebsiella pneumoniae* (4)	+
5. *Shigella dysenteriae* (2)	+
6. *Shigella flexneri* (2)	+
7. *Shigella sonnei* (1)	+
8. *Salmonella heidelberg* (1)	+
9. *Salmonella enteritidis* (2)	+
10. *Yersinia enterocolitica* (5)	+

11. *Proteus mirabilis* (19)	–
12. Proteus vulgaris (7)	–
13. Morganella morganii(6)	–
14. *Pseudomonas aeruginosa* (12)	–
15. Stenotrophomonas maltophilia (4)	–
16. Burkholderia cepacia (1)	–
17. Alcaligenes xylosoxidans (2)	- on TY / + on NBD plate
18. *Haemophilus influenzae* (3)	–
19. Haemophilus parainfluenzae (1)	–
20. Pasteurella multocida (1)	–
21. Mannheimia haemolytica (2)	–

The activity spectrum of albomycin was seemingly more complex in case of gram-positive representatives. While *Streptococcus pneumoniae* was highly sensitive, the closely related *Enterococcus* sp. and even other streptococcal species like *S. pyogenes* and *S. agalactiae* showed complete resistance. Similar was the situation among staphylococci, where *S. aureus* and the non-pathogenic food grade *S. carnosus* was sensitive but *S. epidermidis* showed resistance.

Table 10. Albomycin sensitivity of clinical isolates of gram positive bacterial pathogens.

Pathogenic strains	Albomycin sensitivity
22. Enterococcus faecium (3)	–
23. *Enterococcus faecalis* (3)	–
24. *Streptococcus pneumoniae* (5)	+
25. *Streptococcus agalactiae* (2)	–
26. Streptococcus pyogenes (3)	–
27. *Staphylococcus epidermidis* (3)	–
28. *Staphylococcus aureus* (5)	+
29. *Listeria monocytogenes* (2)	–
30. Bacillus subtilis (2)	+

3.3. *Proteus* does not have a ferrichrome transport system

Although most of the tested *Enterobacteriaceae* were susceptible to albomycin. A total of 26 clinical and lab strains of *Proteus mirabilis* and *P. vulgaris* tested were resistant to albomycin. To avoid possible bias due to strain sampling in restricted area, strains from two different clinics (Tübingen and Ulm) collected at different times were tested.

3.3.1. *Proteus* and *Morganella* resistant to albomycin

Proteus are highly motile and it is possible though less likely that after longer incubation albomycin is exhausted or was diffused away allowing swarming *Proteus* from distal regions to overgrow at the earlier inhibited regions. To rule out false negative results, plates were checked at 2 h. intervals until the plates were covered with a uniform bacterial lawn. No growth inhibition zone around albomycin spots could be observed. To further confirm that swarming was not interfering with the growth inhibition, TY plates were supplemented with either glycerol (1 %) or borate (0.1 %). No growth inhibition zone around albomycin spot was observed.

Table 11. Collection of *Proteus* and *Morganella* strains from different locations.

	Tübingen Klinikum	Ulm Klinikum	Lab repository
Proteus mirabilis	4	11	2
Proteus vulgaris	3	5	1
Morganella morganii	-	-	6

The so-called *Proteus-Morganella-Providencia* group constitutes at present three genera and 10 species. Several of the species are common opportunistic pathogens for humans and other animals. Out of curiosity laboratory strains of *Morganella morganii* (n = 6), which pose less problem with regard to swarming, were checked and found to be resistant to albomycin.

3.3.2. Inability of *Proteus* to utilize ferrichrome as iron source

Being confirmed that *Proteus* is resistant to albomycin, it became obvious to determine why. The possibilities are either or combination of the following 1. Inability of albomycin to cross the membrane barrier *i.e.* no ferrichrome transport system, 2. Inability to activate the antibiotically active moiety *i.e.* lacking functional peptidase, 3. Inability of albomycin to inhibit target site *i.e*. insensitive allele of seryl-tRNA synthetase, 4. Ability to detoxify albomycin by modifying or export. Hypothesis 2-4 is validated only if hypothesis 1 is wrong. To check the hypothesis 1 growth promotion assay with ferrichrome was conducted on *Proteus* under iron-limited condition. Corroborating with the hypothesis 1, ferrichrome couldn't support growth of *Proteus* under iron-limited condition. To further confirm the inability of *Proteus* to uptake ferrichrome, radio labelled iron loaded ferrichrome transport assay was performed with 2 representative strains of *P. mirabilis* and single strain of *P. vulgaris* and compared with *E. coli* (Fig. 10).

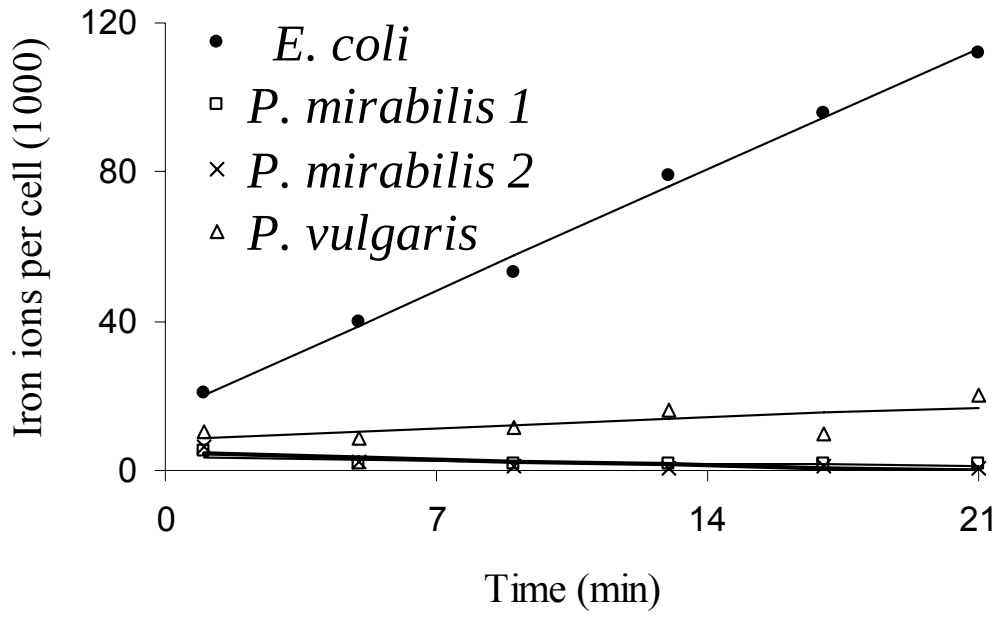


Figure 10. Radiolabelled ferrichrome transport in *Proteus mirabilis* and *P. vulgaris* compared to *E. coli*.

3.3.3. Lack of close ferrichrome transport homologue in *Proteus*

Under standard experimental condition *Proteus* did not show any uptake of ferrichrome. On the other hand most species of *enterobacteriaceae* posses a highly efficient

ferrichrome transport system. To see whether lack of transport is due to absence of ferrichrome transport genes or some tight regulation or an unfavourable mutation diminished the transport below detection level. At that point of time genome sequence information for either *Proteus* sp. in question was not available. To examine the presence of a homologous *fhu*-transport system, genomic DNA-dot-blot of two *P. mirabilis* and one *P. vulgaris* strains were probed with DNA of the *E. coli fhu* region under moderately stringent conditions allowing detection at > 80 % DNA homology. No recognizable signal was observed from *Proteus* spots, though genomic DNA from *E. coli* wild type and a partially deleted *fhu* strain produced spot signals of corresponding intensities.

Figure 11. Dot-blot of chromosomal DNA (in duplicate) from *Proteus* and *E. coli* probed with cloned *E. coli* FhuA gene. An aliquot of the labelled probe used for hybridization was spotted directly on the membrane as reaction control.

3.3.4. In silico search for putative iron-substrate transport systems in *Proteus*

Later availability of *Proteus mirabilis* complete genomic sequence information from microbial genome database of Sanger Institute, UK (yet to be published, annotation in progress) allowed in silico inspection for *fhu* gene homologues. BLASTN search against the whole genome with *E. coli fhuA* DNA resulted in moderate matches over short stretches only. This result supports the interpretation of the dot-blot experiment. Though BLASTX with FhuA protein sequence against translated whole genome sequence revealed two loci with putative ORF's coding for distal FhuA homologues. Unrooted dendrogram made with CLUSTALW multiple alignment (www.align.genome.jp) with experimentally demonstrated

functional outer membrane ferrichrome transport proteins of different gram-negative species (Killmann *et al.*, 1998) and other iron-substrate transporters of *E. coli* (Blattner *et al.*, 1997; Perna *et al.*, 2001) placed *P. mirabilis* proteins Pm1 and Pm2 far from *E. coli* FhuA but closer to FhuE.

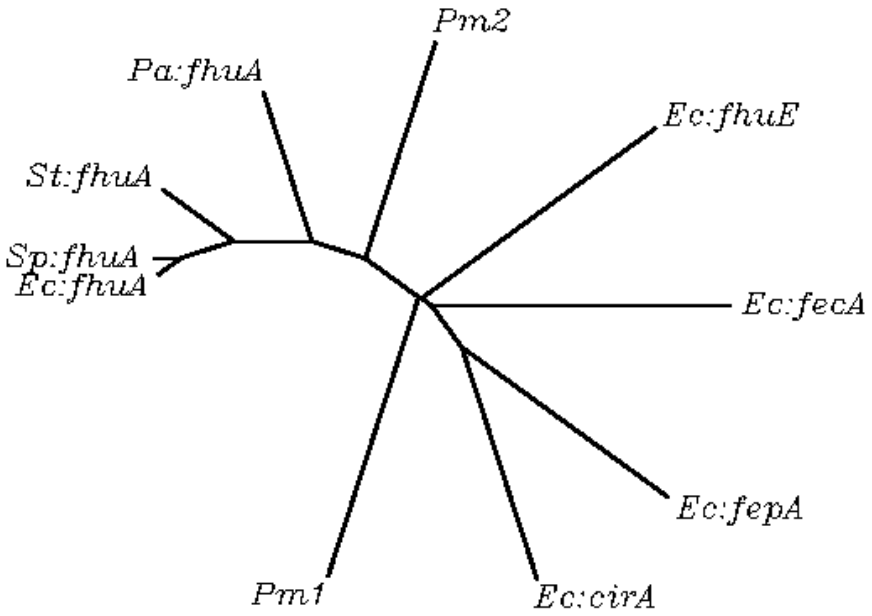


Figure 12. Unrooted dendrogram of experimentally verified outer membrane iron transporters of gram-negative bacteria along with two *Proteus* ORF coding for putative outer membrane iron-siderophore transporter. *Ec; E. coli, Sp; Salmonella paratyphi, St; S. typhimurinum, Pm; Proteus mirabilis, Pa; Pantoea agglomerans*.

BLASTing of protein sequence of *P. mirabilis* putative outer membrane proteins described above, against *E. coli* K-12 yielded poor match with FhuA. Pm1 showed Identities = 166/707 (23%), Positives = 280/707 (39%) at allowed level of Gaps = 68/707 (9%), and Pm2 showed Identities = 266/725 (36%), Positives = 396/725 (54%) at allowed level of Gaps = 54/725 (7%) with *E. coli* K-12 FhuA protein sequence. Substrate specificity of these two putative *P. mirabilis* outer membrane proteins could very well differ from ferrichrome.

3.4. Sideromycin activity against streptococcal species

Streptococcal species can cause a wide range of diseases in animals and humans, including nonsymptomatic commensal like carriage to local infections and even invasive life threatening infection in multiple animals including human. A frequent cause of severe diseases is *S. pneumoniae* (pneumococcus), while closely related viridans streptococci (*S. mitis* and *S. oralis*) are commensals of the human oral cavity. Whereas group A streptococci (GAS; *Streptococcus pyogenes*) commonly known as ‘’flesh eating bacteria’’ can infect irrespective of age, group B streptococci (GBS; *S. agalactiae*) predominantly infect neonates. Among the group C streptococci GCS) and group G streptococci (GGS) are mainly animal pathogens (e.g., *S. zooepidemicus* and *S. canis*). Sensitivity of GAS, GBS and pneumococcus were checked against two hydroxamate sideromycins.

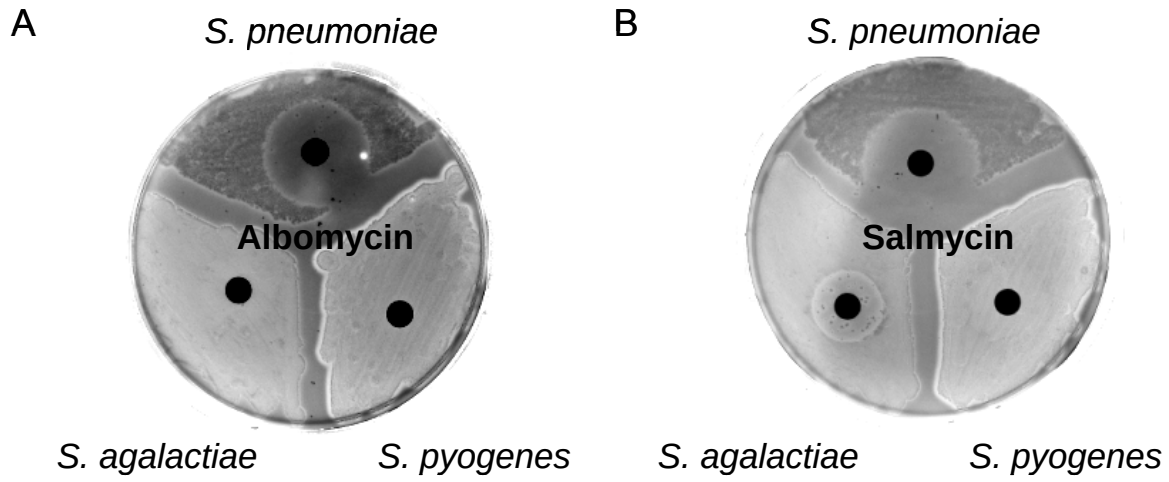


Figure 13. Hydroxamate sideromycin sensitivity among different pathogenic streptococcal species. Growing streptococcal culture was spread on blood agar plates and either albomycin (A) or salmycin (B) was spotted on each species. Growth inhibition zone was observed after 24 h incubation. Alpha-haemolysis of *S. pneumoniae*, beta-haemolysis of *S. pyogenes* and gamma-haemolysis of *S. agalactiae* around the growth zone can be observed.

Different strains (n = 5) of wild type pneumococci were all sensitive to both albomycin and salmycin. GAS strains (n = 3) were resistant to both sideromycins. The GBS strains (n = 2) showed a typical pattern, they were sensitive to salmycin but not to albomycin. The salmycin sensitivity of GBS was ~ 8-10 fold lower than that of unencapsulated

pneumococcus. The dark spots inside the inhibition zone around the sideromycin soaked filter paper discs indicate appearance of spontaneous mutants from sensitive strains.

3.4.1. Evidence of a functional hydroxamate transport system in pneumococcus

Albomycin was found to be a highly effective antibiotic against *Streptococcus pneumoniae* R6 with a minimal inhibitory concentration of 10 ng / ml on blood agar plates. It was surprising to see representative bacterial species from lactic acid group to be so sensitive to hydroxamate sideromycins. Pneumococcus on solid medium was always grown with supplementation of blood. Thus it became necessary to verify the albomycin sensitivity and iron utilization by *S. pneumoniae* R6 without any possible influence of complex enzymatic nature of blood. As pneumococcus or any other lactic acid bacteria not known to produce any siderophore, and the ambiguous reports on the role of iron in the growth of lactic acid bacteria, streptonigrin sensitivity of pneumococci was also checked along with as an indicator of iron uptake.

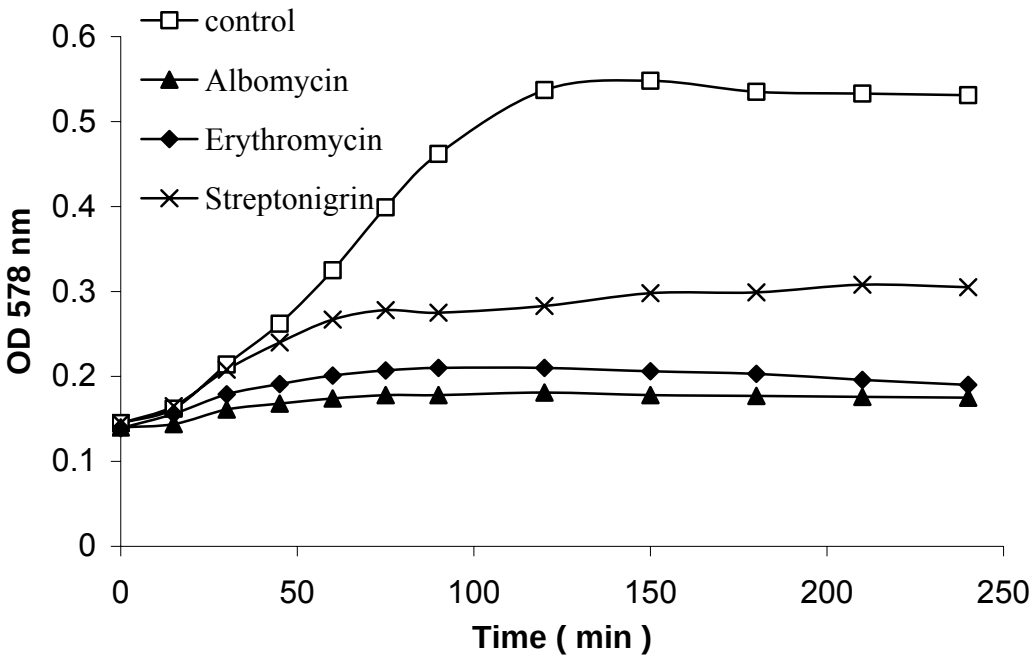


Figure 14. Growth of *S. pneumoniae* R6 in THY broth in presence of different antibiotics including albomycin (2 µg / ml), erythromycin (1µg / ml) and streptonigrin (2 µg / ml).

All the pneumococci tested ceased to grow in presence of albomycin and salmycin as they did with erythromycin when grown in THY broth. Anther antibiotic

streptonigrin which is known to have iron dependent bactericidal activity at low concentration (Braun *et al.*, 1983), considerably inhibited pneumococcal growth.

3.4.2. Hydroxamate siderophores antagonizes sideromycin activity

The very high sensitivity of *S. pneumoniae* could be explained better by active transport than by passive diffusion. In *E coli* ferrichrome transport system recognizes albomycin and mediates the transport inside the cell.

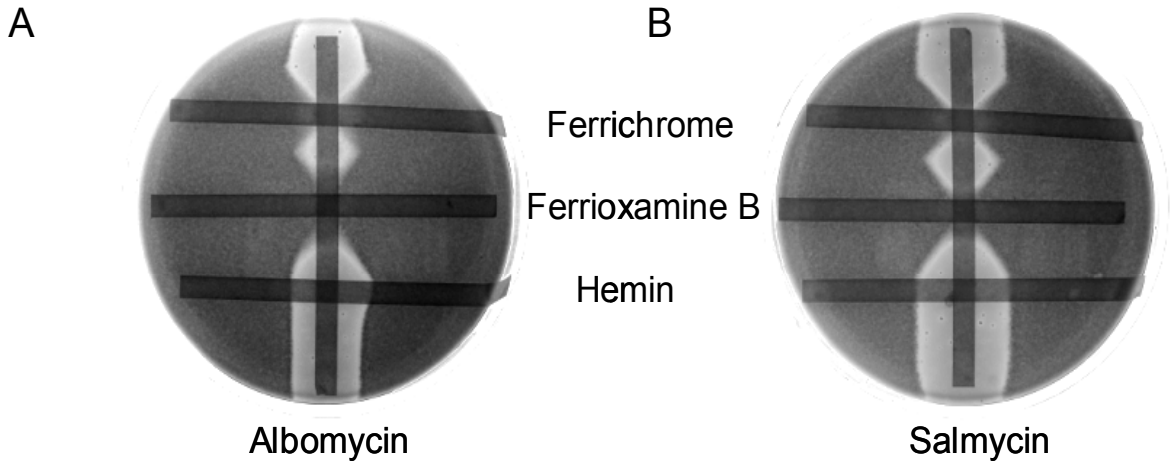


Figure 15. Cross strip assay for competitive antagonism of sideromycin activity by different iron sources. Effect of ferrichrome, ferrioxamine B and heme on the activity of albomycin (A) and salmycin (B). Blood agar plates were seeded with *Streptococcus pneumoniae* R6. A paper strip containing one of the antibiotics was placed on each plate. Paper strips each containing one of the ferric hydroxamates or heme was then placed on the plates at right angles to the antibiotic paper strips as shown. The white areas show growth inhibition by the antibiotics, which do not occur when the ferric siderophores competes for the same uptake system with the antibiotics.

If albomycin is taken up by the same transport system as ferrichrome, ferrichrome should compete with albomycin uptake. This was tested by cross-streaking ferrichrome and albomycin on a plate seeded with *S. pneumoniae* R6. Ferrichrome antagonized the antibiotic action of albomycin. Whereas heme had no effect, another ferric hydroxamate, ferrioxamine B, also inhibited the action of albomycin (Fig. 15A). That

prompted to check the possible antagonistic effect of ferrichrome and ferrioxamine B against another sideromycin, salmycin. Salmycin activity too was competitively antagonized by the presence of both the hydroxamate siderophores but not by structurally unrelated haemin (Fig. 15B). This suggests albomycin, salmycin, ferrichrome and ferrioxamine B, all four ferric hydroxamate compounds, are recognized by very same transport system.

3.4.3. Siderophore part is indispensable for albomycin activity

If both sideromycins and siderophores are the substrate for the very same transport system, it is likely that the transport system recognizes the identical iron co-ordination structure shared by the four transport efficient hydroxamates.

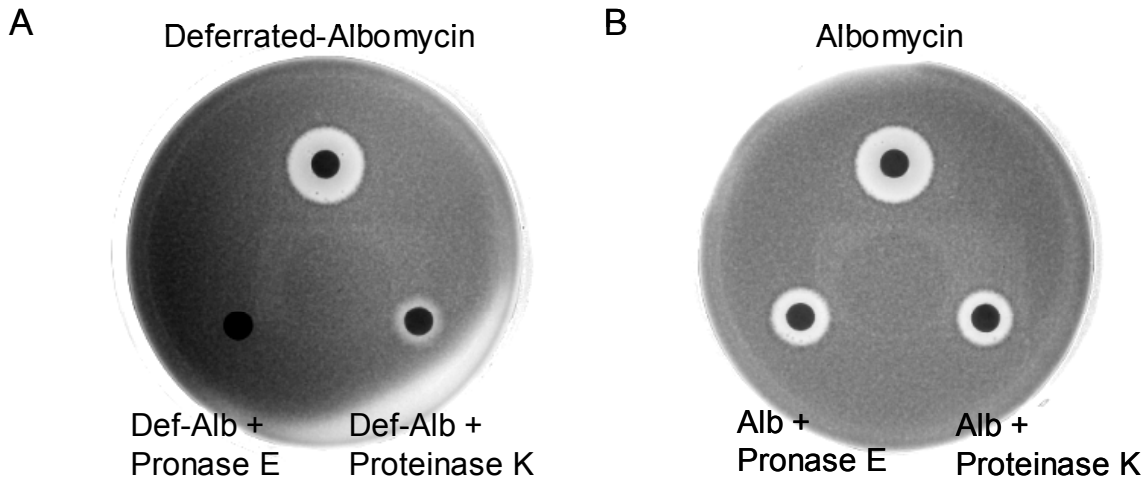


Figure 16. Importance of siderophore moiety for the activity of albomycin against whole cell shown by cleaving albomycin into the antibiotic and siderophore parts with proteinase K and pronase E. Equal amounts of iron loaded or iron free albomycin was digested with either proteinase k or pronase E and spotted on R6 seeded blood agar plates.

As discussed in earlier section, in albomycin the antibacterial moiety is attached to hydroxamate motif via cleavable seryl bridge. In vivo antibacterially active moiety is released by peptidase N. The same reaction can be mimicked in vitro by proteinase K and pronase E. In vitro turn over of this reaction is lower with iron-loaded albomycin as compared to deferrated albomycin. In vivo chelated Fe^{3+} is reduced to Fe^{2+}, which is loosely bound to hydroxamate and can easily be released from albomycin. Release of iron makes albomycin

more vulnerable to peptidase N cleavage resulting in release of the antibiotic moiety inside the cell. Taking advantage of this in vitro cleavage of albomycin at different rates it is possible to define the utility of the hydroxamate part of albomycin in recognition and transport by the R6 pneumococcal cells. Iron loaded and deferrated albomycin was separately digested with either proteinase K or pronase E for 7 days. An equal amount of digested and undigested albomycin was spotted on lawn of sensitive R6 strain and next day growth inhibition was measured. Activity of iron-loaded albomycin subjected to digestion for 7 days decreased to ~ 1/3 of the undigested albomycin (Fig. 16B), whereas activity of digested deferrated albomycin against whole cell reduced to almost undetectable level (Fig. 16A). Digestion of iron loaded albomycin with proteinase K or pronase E for 16 h was not sufficient to produce conceivable decrease in inhibition zone, whereas most of the deferrated albomycin lost activity within 16 h digestion.

3.4.4. Hydroxamate siderophores synergistic to streptonigrin sensitivity

The above results suggest pneumococcus can take up hydroxamate siderophores to cells. If pneumococci utilize hydroxamate siderophores as vehicle of iron transport they should show increased toxicity of streptonigrin in presence of ferrichrome. This was tested by a cross-streak assay on blood agar plates and in THY broth.

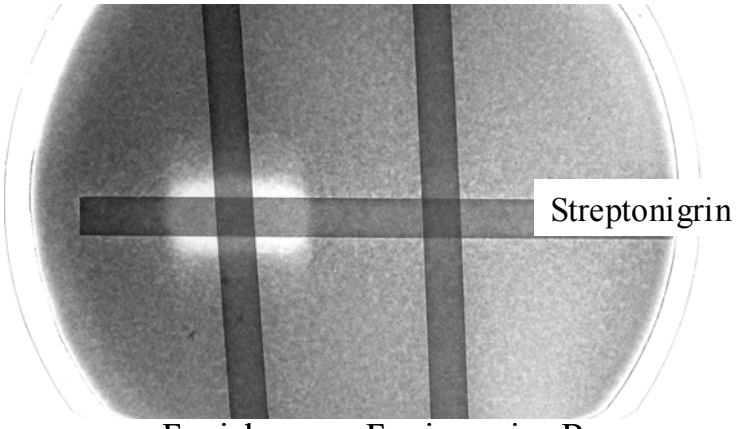


Figure 17. Enhanced bactericidal activity of streptonigrin in presence of ferrichrome against pneumococcus (R6). Paper strips containing ferrichrome or ferrioxamine B were placed at right angles to the streptonigrin paper strips on R6 seeded blood agar plates.

On blood agar plate ferrichrome strongly enhances toxicity of streptonigrin to pneumococci, what can be observed as an inhibition zone around the ferrichrome-streptonigrin junction (Fig. 17). In contrast, ferrioxamine B did not increase streptonigrin sensitivity. The enhancement of streptonigrin toxicity by ferrioxamine can be detected in broth culture, where the effect of ferrichrome is also pronounced (Fig. 40).

3.5. Characterization of ferric-hydroxamate transport loci

To this point observations convincingly indicated the presence of hydroxamate transport system in pneumococci. The next objective was to find the gene/s responsible for ferric-siderophore transport in pneumococcus. Previously no other ferric-siderophore transport genes were reported in pneumococci.

3.5.1. In silico analysis of putative iron-substrate transport systems

Complete annotated genome sequence from two different pneumococcal species R6 and TIGR4 is publicly available from NCBI genome sequence database. A BLAST search of the two annotated pneumococci genomes with ferrichrome binding protein sequences of *E. coli* and *B. subtilis* revealed three loci in both the genomes coding for putative iron transport systems. The Fig. 18 describes the organization and relative orientation of the genes of strain R6.

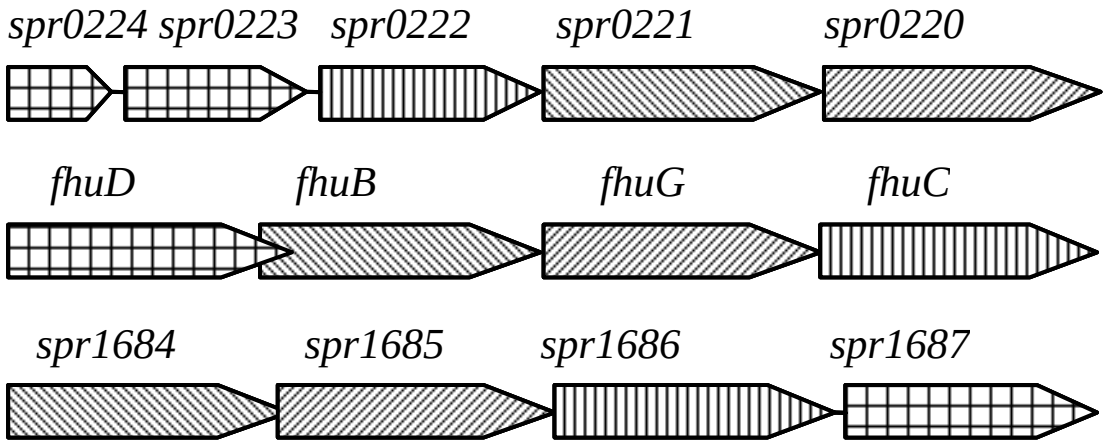


Figure 18. Genetic organization and annotation of putative iron transport cassette of *Streptococcus pneumoniae* R6 with predicted iron transport genes. The identical hatchings indicate predicted related gene functions.

Each of the putative transport operon comprised of 4 components, two membrane spanning permeases, one ATPase and at least one substrate binding lipoprotein.

Table 12. Renamed gene annotation, putative function and predicted size of the protein components of the iron transport operons of R6.

R6 operons	Putative function	Gene annotation	Amino acid residues
spr0220-0224	Substrate binding protein	*spr0224* *spr0223*	64 122
	Membrane permease	*spr0221*	208
	Membrane permease	*spr0220*	344
	ATPase	*spr0222*	363
spr0934-0938	Substrate binding protein	*spr0934, fhuD*	341
	Membrane permease	*spr0935, fhuB*	339
	Membrane permease	*spr0936, fhuG*	335
	ATPase	*spr0938, fhuC*	264
spr1684-1687	Substrate binding protein	*spr1687*	321
	Membrane permease	*spr1684*	318
	Membrane permease	*spr1685*	250
	ATPase	*spr1686*	324

3.5.2. Targeted inactivation of iron transport genes

For targeted mutagenesis it was assumed that one of the iron transport loci was responsible for hydroxamate transport. The putative binding proteins were mutagenized with the notion that their disruption should render cells resistant to albomycin. Targeted gene inactivation in pneumococcus was routinely done by the well described insertion duplication mutagenesis (Berry *et al.*, 1989; Lee *et al.*, 1998; Morrison *et al.*, 1984).

3.5.2.1. Variability of *spr220-spr224* loci

Both the sequenced strain R6 and TIGR4 strains were sensitive to albomycin. In silico analysis of both strains revealed defects in the region corresponding to *spr0220-224* loci. The precise position of the mutations inactivates the transport system. In strain R6 the

substrate binding protein is truncated, while in TIGR4 one membrane permease component is missing. The comparison of the R6 and TIGR4 sequences shows the unmatched *spr220-224* region (Fig. 19).

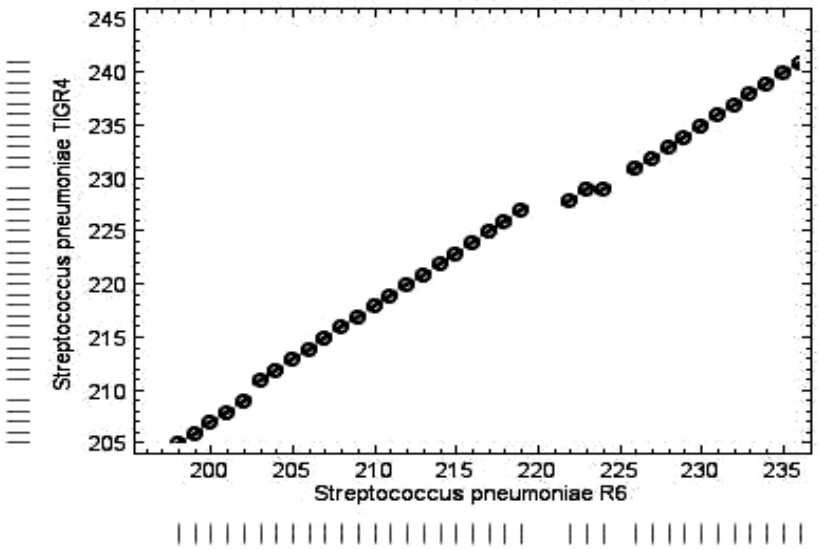


Figure 19. Pair-wise genome comparison of protein homologs (symmetrical best hits) in the two completely sequenced pneumococcus strain R6 and TIGR4. The plot was automatically generated by GenePlot tool hosted by NCBI. Nonalignment in the *spr220-224* loci is evident.

DNA sequence alignment of R6 and TIGR4 for the region corresponding to *spr0220-0224* revealed multiple mismatches (43 over 3447 bp) and a 105 nucleotide long deletion in TIGR4compared to the R6 sequence. The finding that *spr0224/0223* is disrupted in R6 agrees with the observation that many open reading frames for transport proteins are disrupted in *S. pneumoniae* R6 suggesting acquisition of truncated foreign genes or mutations in *S. pneumoniae* of genes which are not required for growth in the fastidious *S. pneumoniae* environment (Bruckner *et al.*, 2004; Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). Since *spr0224/0223* encoded non-functional binding protein *spr0220-0224* was not studied further.

3.5.2.2. Involvement of *fhuDBGC* loci

An internal fragment of *fhuD* was PCR amplified from the *S. pneumoniae* R6 genomic DNA with the primer pair AATACT*TCTAGA*GAGCATGCGCCTG and GTTG*GAATTC*ATGAGGCTGCTAACG each having one unique restriction enzyme recognition site (marked in italics). Digested PCR product was cloned between the unique

EcoRI-XbaI sites of pJDC9 in *E. coli* DH5α. Plasmid with the right insert was confirmed by sequencing and designated pAPD1. pAPD1 was purified from *E. coli* and transformed into *S. pneumoniae* R6. Erythromycin resistant colonies were picked and the insertion site was confirmed by PCR and by direct sequencing with outward primers specific to the plasmid sequence flanking the cloning site. A mutant with the desired insertion in *fhuD* was designated API1.

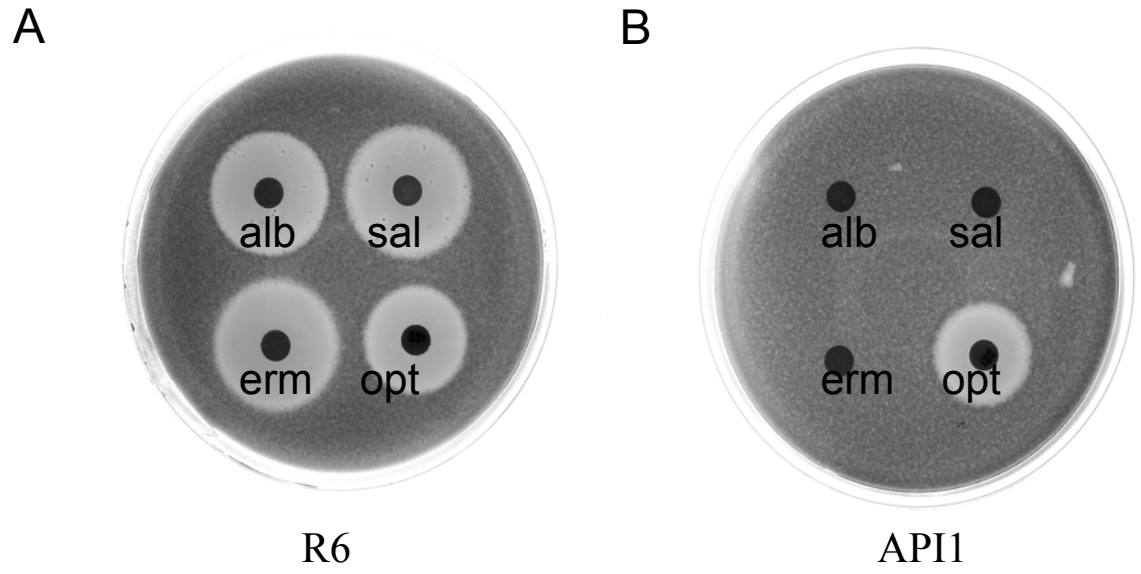


Figure 20. Sideromycin sensitivity of the strain R6 and API1 *fhuD*. Optochin sensitivity is specific for pneumococci and erythromycin is the resistance marker used for insertion duplication mutagenesis.

The *fhuD* mutation resulted in albomycin resistance (Fig. 20). Since *fhuD* is the foremost transcribed component in the operon, its inactivation is likely to have a polar effect on the transcription of the downstream genes coding for permeases and ATPase component of the machinery (Fig. 30). While performing IDM (Insertion duplication mutagenesis) of *spr0934* caution was taken to orient the *lac* promoter of pJDC9 just upstream of truncated *spr0934* followed by *spr0935-0938*. This may allow independent expression of *spr0935-0938*, if the *lac* promoter (derived from *E. coli*) is able to direct the desired transcription in pneumococci. But the possibility of inactivation of the downstream *fhuBGC* genes of the operon ruled out, making it difficult to interpret the role of *fhuD*.

3.5.2.3. Albomycin sensitivity independent of functional *spr*1687

spr1687 is another putative binding protein and was inactivated by IDM to construct strain API2.

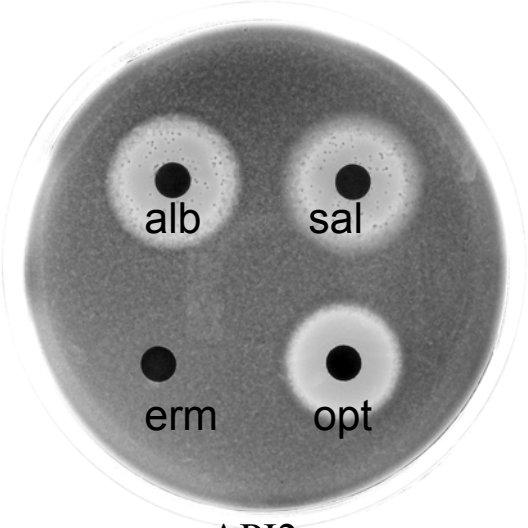


Figure 21. Sideromycin sensitivity of *spr*1687 mutant API2. Optochin is presumptive test for pneumococcal identification and erythromycin is the resistance marker used for insertion duplication mutagenesis.

An internal fragment of *spr1687* was PCR amplified from the *S. pneumoniae* R6 genomic DNA with the primer pair AGCTC*GGATCC*AACAGAGATAACC and TGATT*GAATTC*CGCCTCCGCTTAG and cloned between BamHI- EcoRI of pJDC9 to produce the disruption plasmid pAPID2. pAPID2 was transformed into *S. pneumoniae* R6 to create API2 with an insertion in *spr1687*. Disruption of *spr*1687 did not considerably reduce albomycin sensitivity (Fig. 21). Since *spr1687* is the last gene in the predicted operon, its elimination has no downstream effect (Fig. 18). In a recent report (Tai *et al.*, 1991) *spr*1687 gene product was found to bind haem and haemoglobin. In streptonigrin toxicity assay API2, *spr*1687$^-$ strain showed strong growth inhibition in presence of ferrichrome and ferrioxamine, as wild type R6 (Fig. 40). These results conclusively rules out any role of *spr*1687 gene product in hydroxamate transport.

3.5.3. Strain with deletion in substrate binding protein developed

To confirm the FhuD activity in antibiotic sensitivity and to examine its role in ferric hydroxamate transport, an *fhuD* deletion mutant strain APD1 was constructed. This mutant APD1 showed resistance to albomycin and salmycin (Fig. 22).

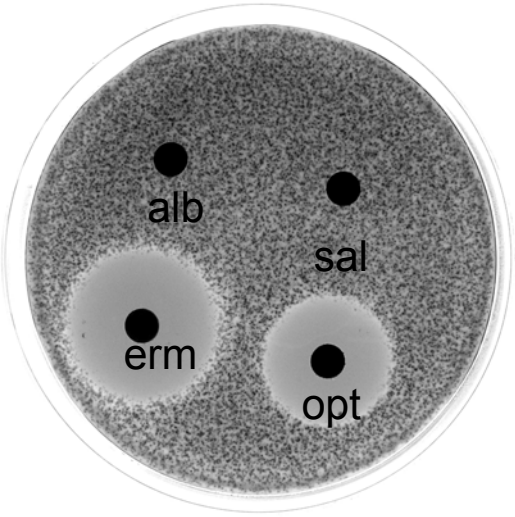


Figure 22. Sideromycin sensitivity of APD1 Δ *fhuD*. Optochin sensitivity identifies pneumococci and erythromycin is the resistance marker used for insertion duplication mutagenesis.

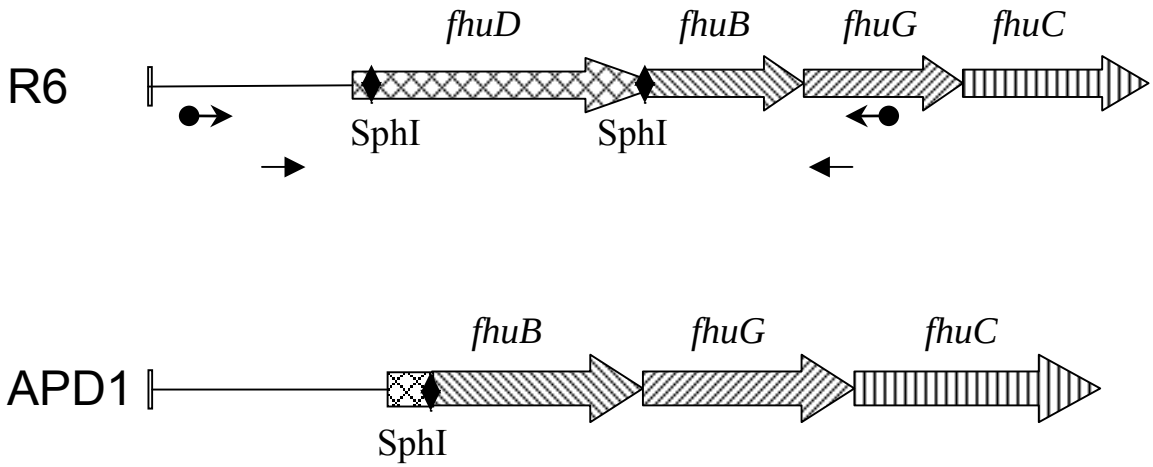


Figure 23. Schematic representation of the construction of deletion mutant APD1. Different arrows (solid) indicate the positions of primer pair used to amplify the *fhu* region. Hollow arrow indicates transcription promoter of the operon.

For creating APD1 Δ *fhuD*, the 4.5 kb region covering the *fhuD* sequence was PCR amplified from genomic DNA with the primers TGCTTGAACTTGCTTGTTGG and TTCAACATTGGCCTTAACCA. In silico restriction analysis of the *fhu* region sequence of *S. pneumoniae* R6 revealed two native SphI restriction sites. The fragment was digested with SphI, which cleaves 105 bp downstream of the start codon and 16 bp upstream of the stop codon of *fhuD* to yield three fragments of 0.9, 1.2 and 2.4 kb. The 1.2 and 2.4 kb fragment was gel purified and ligated resulting in a 904 bp deletion in *fhuD*. This ligation mixture served as the template for nested PCR with the primers AGCTATGGCAGGACTTACAAC and GACCACGGCTTACAAGATCAG to amplify a 3 kb region encompassing the *fhuD* deletion. The PCR product was gel purified and transformed into *S. pneumoniae* R6. Transformants were selected with streptonigrin in the presence of ferrichrome. Individual colonies were checked by PCR for the deletion. A colony designated APD1 showing a 0.9 kb shorter sequence than wild type in the *fhu* region was selected for further study.

3.5.4. Unbiased mutagenesis screen identified permease components

From the results obtained from targeted mutagenesis of two binding proteins of two putative iron transport operons it was concluded that *fhuD* is an essential component of hydroxamate transport, while *spr1687* was dispensable for hydroxamate transport. In gram-positive bacteria binding proteins are not always encoded next to the genes for ABC transport proteins across the cytoplasmic membrane. For example, *S. aureus* encodes two *fhuD* genes that are not linked to *fhuCBG* (Cabrera *et al.*, 2001; Sebulsky and Heinrichs, 2001). To find out the permease and ATPase components of the transport system in question, unbiased random mutagenesis with EZ-Tn5 transposon was performed. Library construction is presented as schematic drawing in Fig. 24. The method took advantage of the ability of pneumococcus to take up linear DNA. Initial transposition of an *erm*B cassette on pneumococcal chromosomal DNA was performed in vitro with purified transposase (available from Epicentre). Gaps and nicks were repaired in vitro, and then the mutations were transferred to *S. pneumoniae* R6 by natural transformation. Clones with transposon insertion recovered by plating transformation mixture on blood agar plates containing 1 mg/l erythromycin.

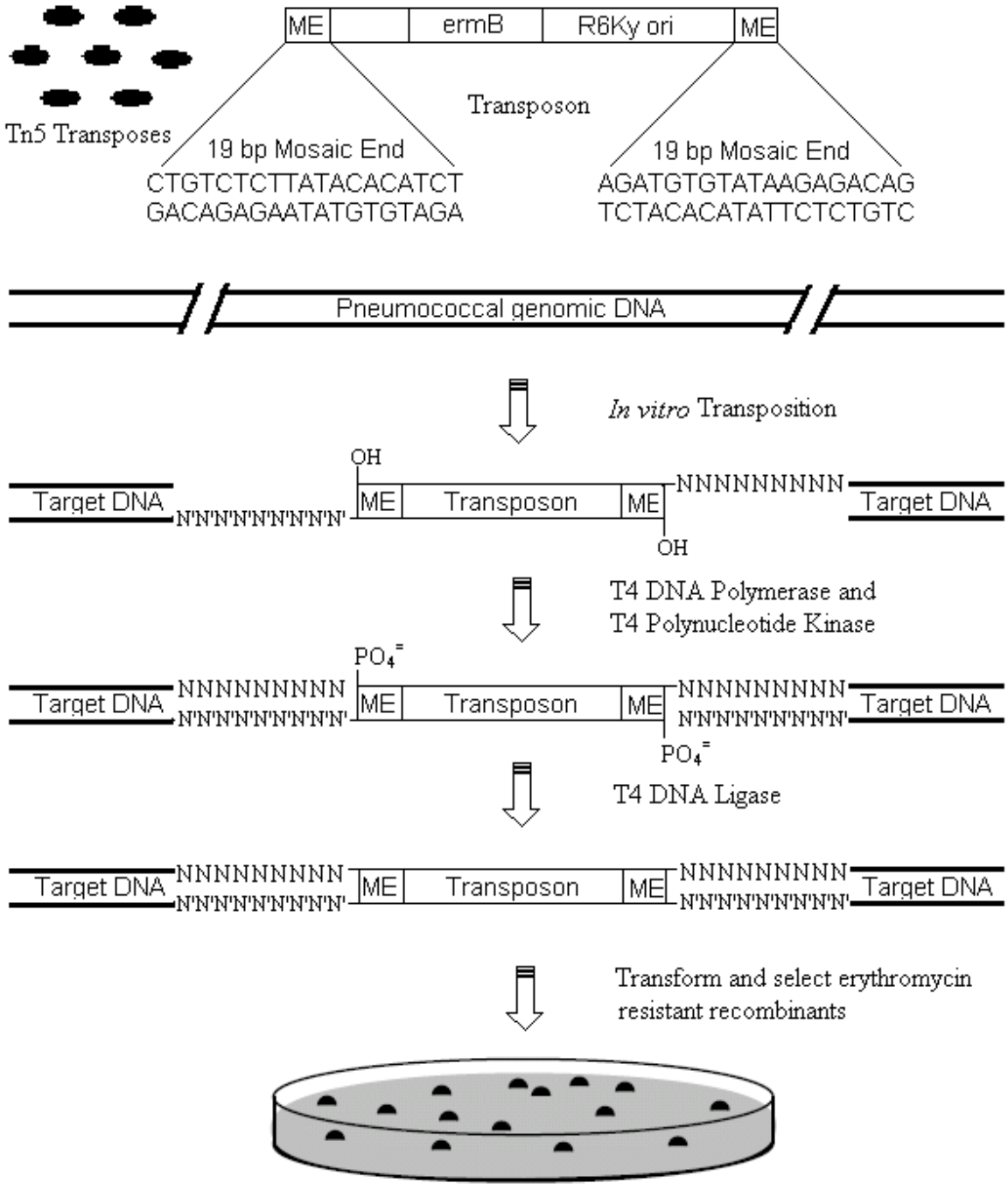
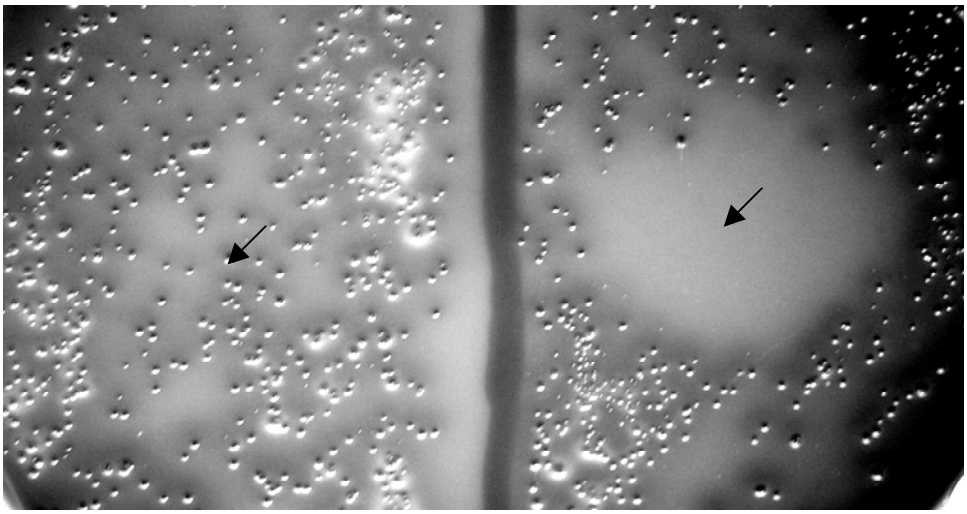


Figure 24. Schematic representation of in vitro random mutagenesis in R6 by Ez-Tn5 transposon.

Erythromycin resistant transformants were pooled to yield a Tn5 insertion library and saved as glycerol stock. The library was screened for albomycin resistant mutants. To confirm that the albomycin resistance derived form the transposon insertion and not from

spontaneous mutations, genomic DNA was isolated from colonies which were both albomycin and erythromycin resistant and transformed back into wild type R6. For quick viewing the co-selection, transformation mixture was directly spread on blood agar plates under erythromycin selection and albomycin was spotted on the lawn. In case of co-selection of the erythromycin-albomycin resistance marker all the erm^R colonies will also be albomycin resistant, showing no inhibition zone around the albomycin spot (Fig. 25). If the markers segregate due to two independent mutations at different loci, albomycin should give an inhibition zone, and the number of resistant mutants will indicate physical distance between transposon insertion site and the spontaneous mutation in albomycin transport gene/s. For more accurate quantitative determination of the co-selection *vs.* segregation, equal amounts of each transformation mixture were examined for erythromycin resistance and erythromycin-albomycin double resistance. Similar numbers of transformants on the single and double antibiotic plates indicated co-selection of the resistance traits confirming albomycin resistance by Tn*5* *ermB* insertion.



Coselection Segregation

Figure 25. Co-selection and segregation of resistance to erythromycin and albomycin is shown. Genomic DNA from the test clone was transformed into wild type R6. After a 4 h recovery growth period in THY broth, transformants were spread on blood agar plates with erythromycin. Albomycin was spotted (shown by black arrow) on each transformants. An inhibition zone is indicative of segregation of the double resistance traits.

Two such mutants, APT1 and APT2, were isolated and the Tn*5* insertion sites were determined by sequencing with transposon specific outward primers. Mutants, APT1 and APT2 contained EZ-Tn*5* insertions in *fhuB* and *fhuG* (Fig. 30), respectively, encoding polypeptides forming the predicted transmembrane transporter adjacent to *fhuD*. The *fhu* fragment of mutants APT1 and of APT2 was 1391 bp longer than the *fhu* fragment of the wild-type strain R6 (Fig. 29). Both mutants were also resistant to salmycin (Fig. 26), confirming single transport system for both hydroxamate sideromycins. Since these were the only selected mutants, it is likely that FhuD is part of the FhuBGC transporter.

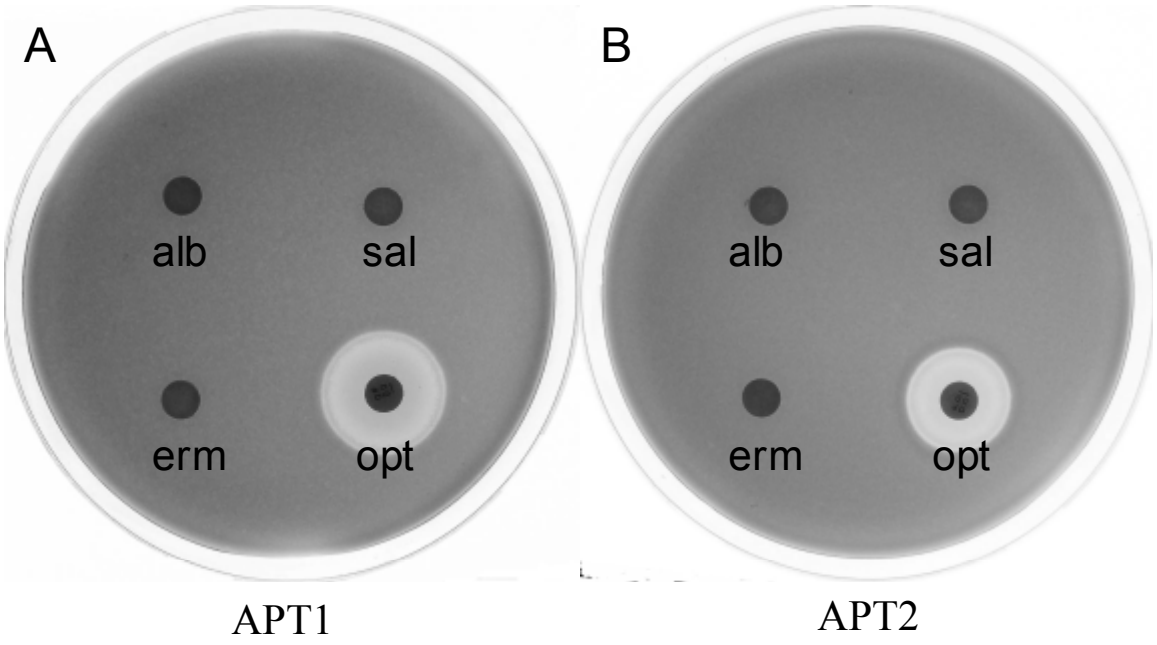


Figure 26. Sideromycin sensitivity of APT1 *fhuB* and APT2 *fhuG*. Optochin is specific for pneumococci and erythromycin is the resistance marker used for insertion duplication mutagenesis.

3.5.5. Complementation in trans restores hydroxamate transport

APD1 Δ*fhuD* was complemented by inserting *fhuD* into the chromosomal *malMP* region. The C-terminal fragment of *malM* was PCR amplified with the primer pair CTT*GAGCTC*TTTGCTGAGTATA and GATAA*CATATG*TAGTTGTCTCCTG and *fhuD* with the primer pair GTTTAAGGAGTT*CATATG*AAGAACAA and TGTG*TCTAGA*CCGAGTATACCTGGA. The *malM* product was digested with SacI and NdeI, and the *fhuD* product with NdeI and XbaI. Both products were gel purified and a three-

way-ligation was performed with a SacI-XbaI fragment of pJDC9. The resulting plasmid pAPIC was purified from *E. coli*. Insertion duplication at the *malM* region introduced *fhuD* downstream of *malM* resulting in strain APD1CI. Construction of APD1CI schematically presented in Fig. 27.

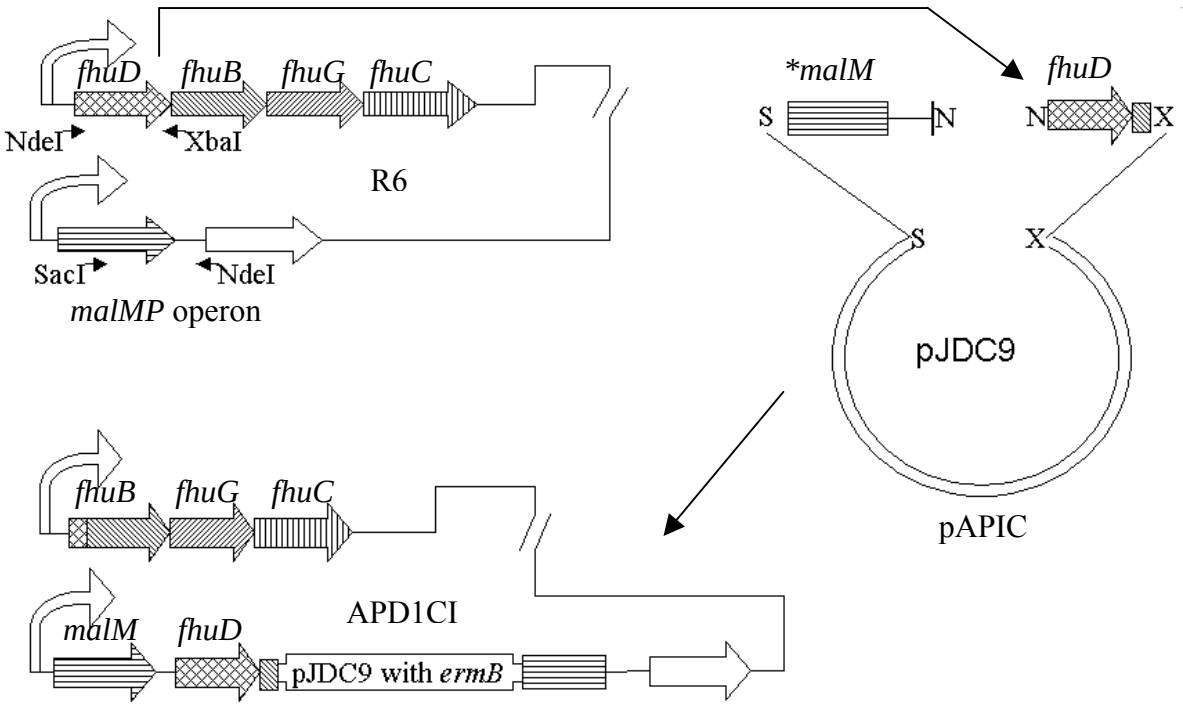


Figure 27. Schematic representation of construction of APD1CI complemented strain. Complete *fhuD* and the C-terminal fragment of *malM* were amplified from R6, cloned in the right orientation determined by the introduced SacI-NdeI-XbaI restriction sites. Resultant plasmid pAPIC was transformed into APD1 to create APD1CI by ectopic insertion of *fhuD* in *malMP* operon.

The trans-complemented strain APD1CI regained sensitivity to albomycin and salmycin. This confirmed the involvement of *fhu*D in hydroxamate transport (Fig. 28A). In APD1CI, *fhuBGC* is being transcribed from the native promoter, whereas *fhuD* is transcribed from the maltose regulated *malMP* operon (Nieto *et al.*, 1997). To check whether maltose induction of *fhuD* expression can induce transport, sideromycin sensitivity of APD1CI in the presence of maltose was examined. Maltose slightly though not profoundly increased sensitivity to albomycin, presumably because maltose positively regulates transcription of the

malMP operon from where *fhuD* is expressed (Fig. 28B). Apparently, the FhuD step is rate limiting in sideromycin transport.

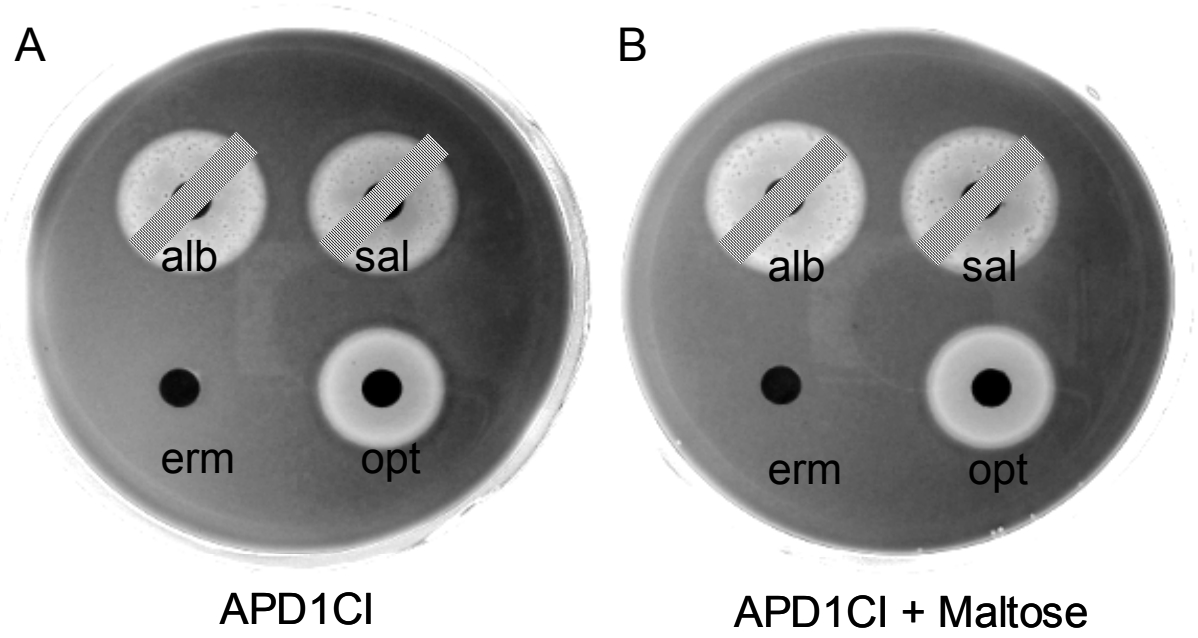


Figure 28. Sideromycin sensitivity in trans complemented strain APD1CI. Expression of *fhuD* under maltose regulation. Sideromycin sensitivity increases in presence of maltose when same inoculum was used to seed blood agar plate (A) and blood agar plate supplemented with maltose (B).

3.5.6. Genotyping of the recombinant strains

Genetic organization at *fhu* loci was confirmed by PCR and sequencing in wild type and transport negative mutants. Genomic DNA of the *fhu* locus from upstream of *fhuD* to downstream of *fhuG* was PCR amplified with primer pair GACCACGGCTTACAAGATCAG and AGCTATGGCAGGACTTACAAC. Transposon insertion mutants APT1 and APT2 yielded PCR products ~ 1.5 kb larger than the wild type, whereas deletion mutant APD1 and the complemented strain produced bands of ~ 1 kb smaller than R6 strain (Fig. 29). Restriction analysis showed that unlike R6 (2 SphI I site) *fhu* loci of APD1 and APD1CI contain only one SphI I restriction site. From the restriction analysis and sequencing organization of *fhu* loci was determined as shown in Fig. 30.

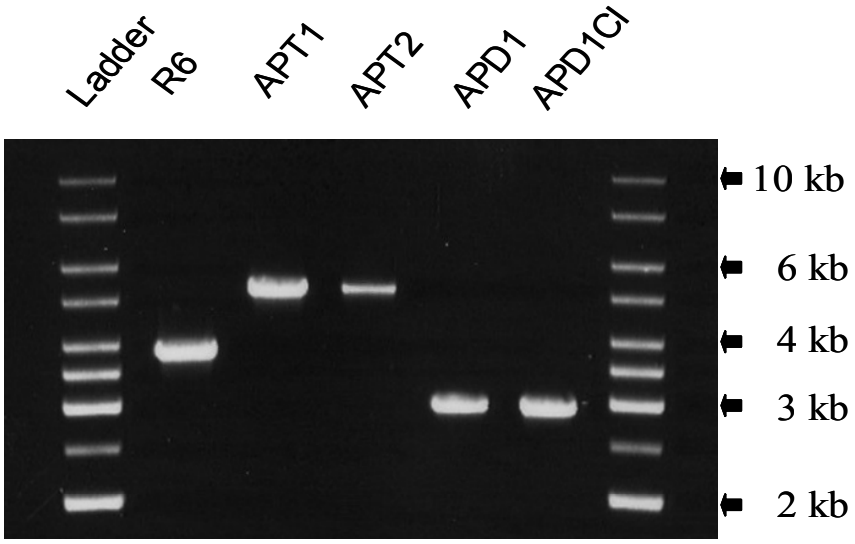


Figure 29. DNA fragments obtained by PCR of the entire *fhu* operon of *Streptococcus pneumoniae* R6, and mutants APT1, APT2, APD1, APD1CI.

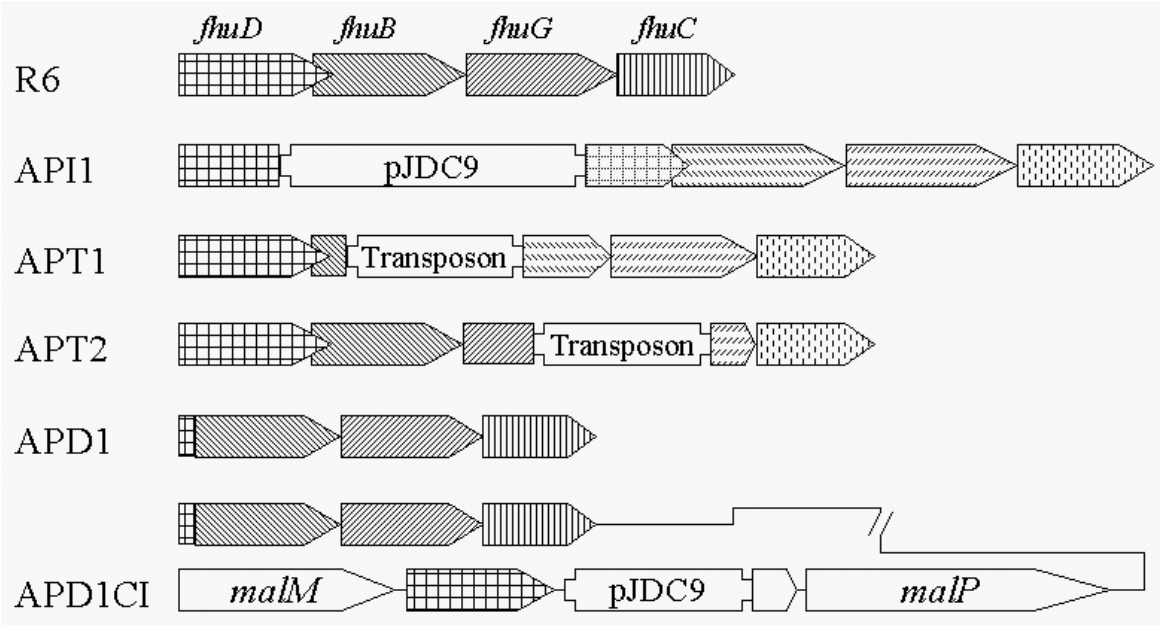


Figure 30. Arrangement of the *fhu* genes of *S. pneumoniae* R6 and the *fhu* mutants constructed in this study. Mutants APT1 *fhuB* and APT2 *fhuG* were constructed by in vitro Tn*5* mutagenesis of the entire genome, and mutant API1 *fhuD* was constructed by insertion duplication mutagenesis of the chromosome with a derivative of plasmid pJDC9 encoding an internal *fhuD* fragment. APD1 *ΔfhuD* contains an internal deletion in *fhuD*, and APD1CI is a derivative of APD1 in which *fhuD* was cloned downstream of *malM*. The different hatchings of the *fhu* genes downstream of the mutated gene indicate predicted polar effects on the downstream gene transcription.

3.6. Analysis, cloning and substrate specificity of binding protein

Genetic analysis revealed that FhuD is a necessary component of hydroxamate transport. This protein was further characterized.

3.6.1. Primary sequence analysis of Sp-FhuD

The two pneumococcus genomes (R6 and TIGR4 strains) posses the identical *fhu* loci with a 341 amino acids long FhuD protein that shows 100% sequence identity in both strains.

MKNKFFLIAILAMCIVFSACSSNSVKNEENTSKEHAPDKIVLDHAFGQTILDKKPERV
ATIAWGNHDVALALGIVPVGFSKANYGVSADKGVLPWTEEKIKELNGKANLFDDLD
GLNFEAISNSKPDVILAGYSGITKEDYDTLSKIAPVAAYKSKPWQTLWRDMIKIDSKA
LGMEKEGDELIKNTEARISKELEKHPEIKGKIKGKKVLFTMINAADTSKFWIYTSKDP
RANYLTDLGLVFPESLKEFESEDSFAKEISAEEANKINDADVIITYGDDKTLEALQKDP
LLGKINAIKNGAVAVIPDNTPLAASCTPTPLSINYTIEEYLNLLGNACKNAK

Figure 31. Sequence of FhuD. Nonpolar amino acids are shown in green, polar amino acids in blue, aromatic amino acids in red and sulphur containing amino acids in yellow. Abundance (7.6%) of aromatic amino acids is evident.

The theoretical molecular weight of the whole protein is 37.5 kD with a pI of 5.38 which corresponds to -7 net negative charges of the protein. Grand Average hydropathicity of FhuD is negative (GRAVY, -0.274), which indicates that the protein is hydrophilic and likely be soluble (Kyte and Doolittle, 1982). Functional homologues of free moving periplasmic binding protein of gram negative bacteria are usually anchored to the cytoplasmic membrane by an lipophillic post-translational modification in case of gram positive bacteria (Antelmann *et al.*, 2001; Dwyer and Hellinga, 2004; Felder *et al.*, 1999; Quiocho and Ledvina, 1996; Sutcliffe and Harrington, 2002).

Analysis of the signal sequences of different lipoproteins revealed common structural features that are recognized prior to lipid modification. The most important of all is the presence of a distinct 4 amino acid long sequence, referred to as lipobox within the first 40 residues from the N-terminus with the consensus sequence [LVI][ASTVI][GAS][C]. Apart

from the lipobox, the N-terminal 5-7 residues with positively charged Lys or Arg residues mostly at the 2nd position and an intervening stretch of hydrophobic and uncharged residues of 7-22 amino acid length (Antelmann *et al.*, 2001; Babu *et al.*, 2006; Edman *et al.*, 1999; Madan Babu and Sankaran, 2002; Navarre *et al.*, 1996; von Heijne, 1989). FhuD N-terminus presents the characteristics of a lipoprotein (Fig. 32) with cutting site between FSA↓C20 with a putative lipid modification site at Cys20.

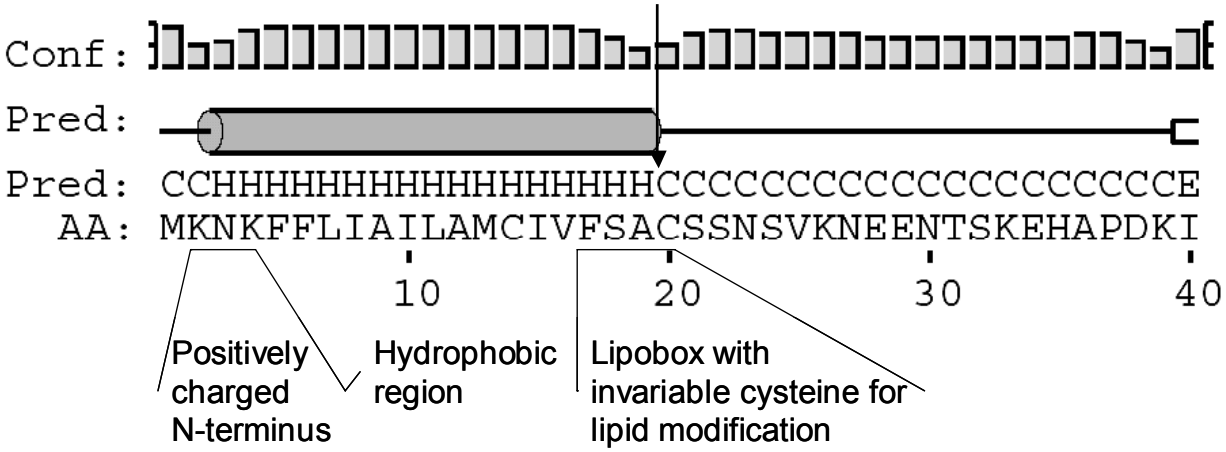


Figure 32. Identification of a lipoprotein signal in the N-terminus of Sp-FhuD.

3.6.2. Cloning, overproduction and purification of the binding protein FhuD

Complete *fhuD* was PCR amplified from genomic DNA with the primer pair GTTTAAGGAGTT*CATATG*AAGAACAA & TAAGACT*GGATCC*TGTGTTTATACCGA. The PCR product was digested with NdeI and BamHI and ligated into pET28a digested with NdeI and BamHI. The resulting plasmid pAPBP encoded FhuD fused at the N-terminal end to a 20 residue peptide which contains a sequence of 6 histidine residues for purification on a Ni-NTA agarose column. The *fhuD* derivative was initially cloned in *E coli* DH5α and then transformed into *E. coli* BL21 (DE3). *E. coli* BL21(DE3) pAPBP was grown with vigorous shaking at 37°C in TY medium supplemented with kanamycin (30 μg/ml) until an OD_{578} of 0.6 was reached. Over-expression of *fhuD* was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG) in the grow culture (Fig. 33).

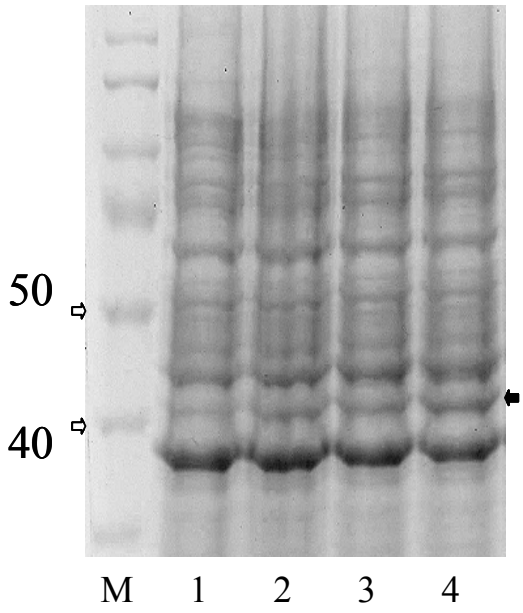


Figure 33. Overexpression of His_6-FhuD. M; protein molecular weight marker, lane 1; uninduced, lane 2; one hour after induction, lane 3; two hours after induction, lane 4; three hours after induction. Arrow indicates overexpressed His_6-FhuD protein.

3.6.3. Ligand binding specificity of FhuD

Primarily binding proteins determine the substrate specificity of bacterial ABC importers. To examine whether FhuD functions as a binding protein, FhuD was isolated and purified. The *fhuD* gene was cloned in plasmid pET-28a that resulted in a protein with six histidine residues at the N-terminal end. Synthesis of $(His)_6$FhuD in *E. coli* was induced by 1 mM IPTG and the protein was purified by affinity chromatography on a Ni-NTA agarose column. Binding of the ferric hydroxamates and heme was examined by protection of $(His)_6$FhuD against proteolytic digestion by added proteinase K.

This assay demonstrated substrate binding to the *E. coli* FhuD protein, whose proteolysis is inhibited by cognate substrates (Koster and Braun, 1990; Rohrbach *et al.*, 1995). This was also the case with FhuD of *S. pneumoniae* which was completely degraded by protease K in the absence of substrate (Fig. 34**,** lane 9) and truncated to a smaller, stable product in the presence of ferrichrome, ferrioxamine B, albomycin and salmycin (Fig. 34, lanes 3, 4, 7, 8) but not in the presence of heme (Fig. 34, lane 6), another possible iron source for *S. pneumoniae*, or $FeCl_3$ (Fig. 34, lane 5). The assay depended on the resistance of ferrichrome and albomycin to proteinase K. Iron-loaded albomycin used in the assay was

resistant since the activity was not decreased but iron-free albomycin was degraded (Fig. 16). Iron coordination renders albomycin protease resistant even in the seryl bridge between the iron centre and the antibiotic that is not involved in iron binding.

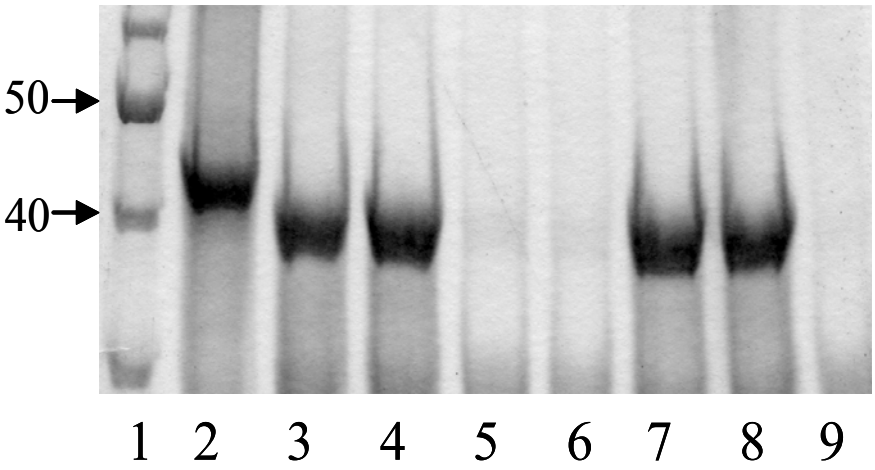


Figure 34. Proteolytic digestion of $(His)_6$-FhuD by proteinase K in the absence (lane 9) and presence of ferrichrome (lane 3) , ferrioxamine B (lane 4), heme (lane 6), albomycin (lane 7), salmycin (lane 8) and $FeCl_3$ (lane 5), untreated $(His)_6$-FhuD (lane 2), and molecular size markers (lane 1).

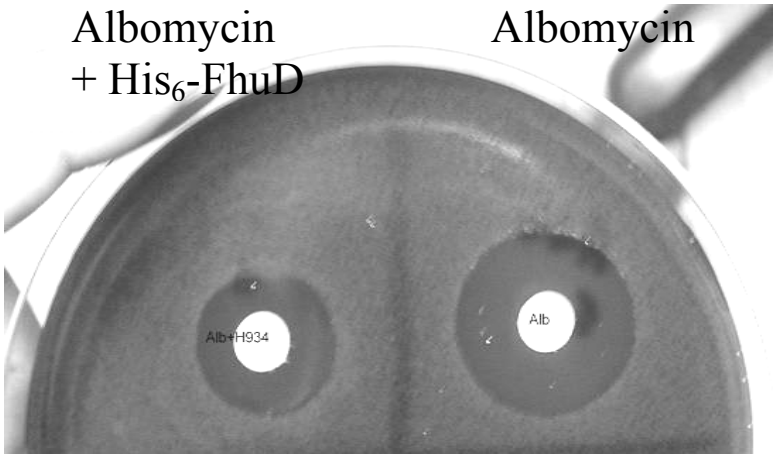


Figure 35. Equal amount of albomycin with or without incubation with purified recombinant His_6-FhuD was spotted on R6 seeded blood agar plate. Presence of recombinant FhuD leads to decrease in the size of the inhibition zone, indicating binding of albomycin by the recombinant protein but inefficient delivery to the membrane permease, making bound albomycin unavailable for uptake.

In another assay, isolated FhuD was incubated with albomycin, and the antibiotic activity of albomycin was tested on plates. FhuD reduced albomycin activity (Fig. 35), which suggests that binding to FhuD decreases the free albomycin concentration available for entering the cells. It also shows that added FhuD cannot functionally contact FhuB and FhuG and deliver albomycin to the transport system.

3.6.4. Tertiary sequence analysis; homology modelling of Sp-FhuD

In Gram-negative bacteria, periplasmic binding proteins (PBPs) capable of binding diverse nutrients act as a shuttle between the cognate transporters or chemotaxis receptors between inner and outer membranes. Three-dimensional structures of many PBPs with diverse binding specificities have been resolved to atomic resolution with the use of X-ray crystallography, both in the free and liganded state. Almost by rule PBPs consist of two large lobes that captures the bound ligand in a closed or semi-closed way, resembling a venus flytrap model.

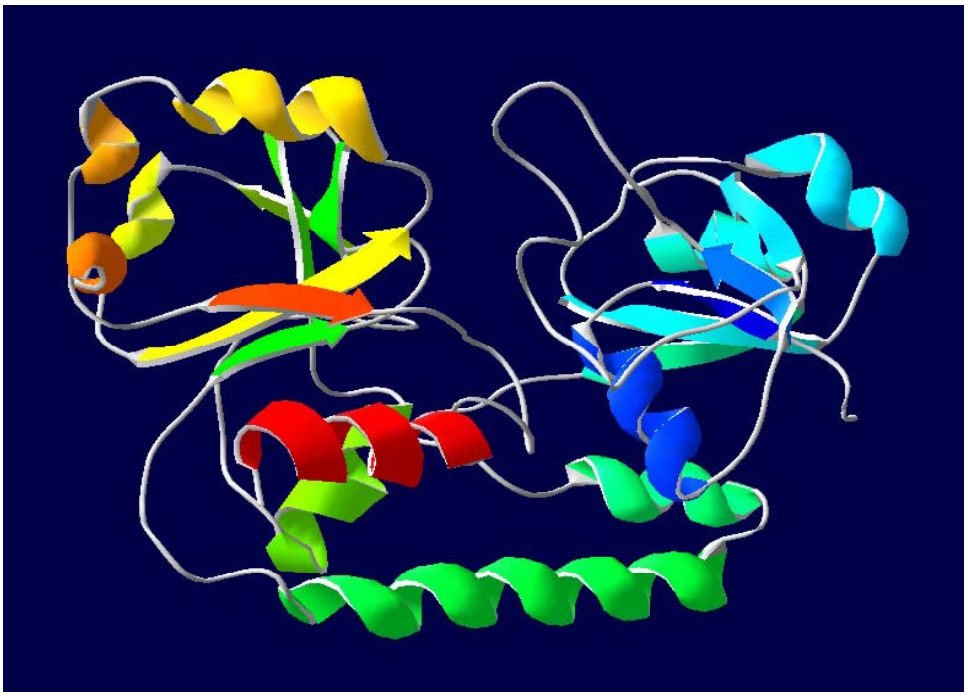


Figure 36. A 3D model of *S. pneumoniae* FhuD has been built using the 3D structure 1EFD chain 'N' (*E. coli* FhuD) protein as template. This template shares 20.6% identities with the Sp-FhuD sequence (using the ALIGN program). From the N-terminal to the C-terminal end the colour gradient is from blue-green-yellow-orange-red. The automated three-dimensional structure is built using the modelling package MODELLER hosted by ESyPred3D server.

In the gram positive bacteria which lack outer membrane and periplasmic compartments, the binding proteins of similar nutrients are homologous to genes encoding gram-negative PBPs, with an additional lipid anchor to the cytoplasmic membrane Together with the extensive information available on the mechanism of ligand binding to PBPs, such models can serve as a template for constructing close to reality 3D models of homologues proteins whose crystal structure have not or could not been solved. Though many PBPs structures have been solved from gram negative bacteria there is lack of resolved structures of their homologues in gram-positive bacteria. Gram positive binding proteins are expected to have similar lobular arrangements as their PBP homologues. 3D homology modelling is not sufficient to predict accurate structure, specially for regions with lower sequence similarity. Nevertheless, such models are useful tools for the design of a rational mutagenesis.

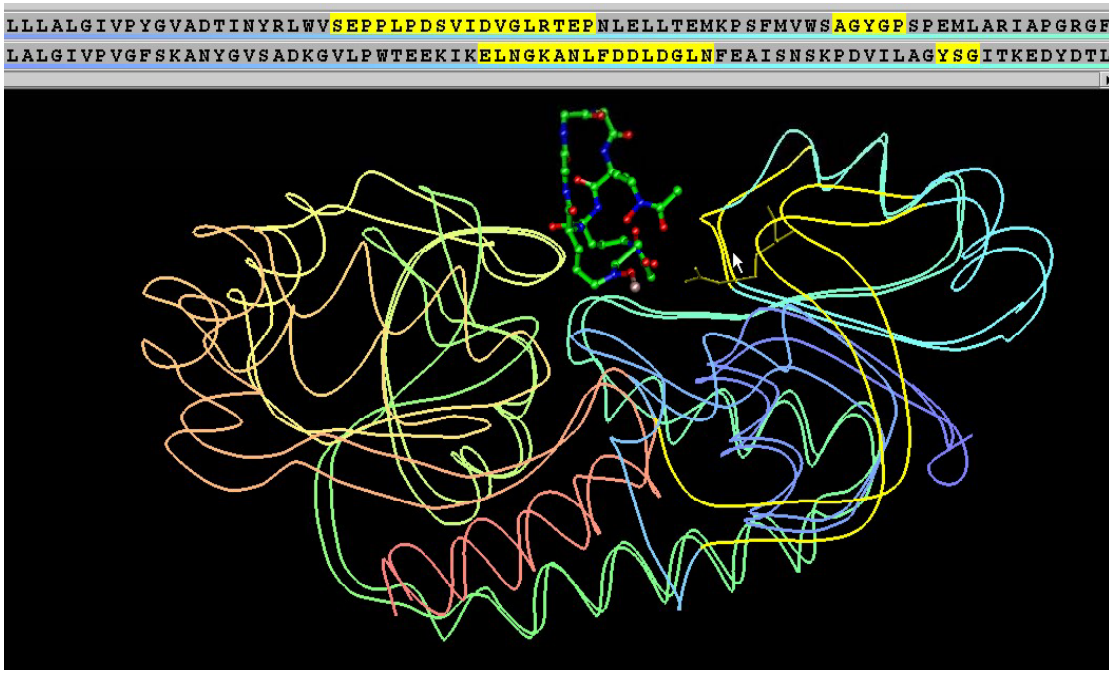


Figure 37. Structural overlay of 3D homology model of Sp-FhuD and *E. coli* FhuD (PDB entry 1EFD) using Ligand Explorer. Arrow (white) indicates position of Y106 of Ec-FhuD and the corresponding Y133 of Sp-FhuD at the binding cleft, also highlighted in yellow in the primary sequences in the upper panel. Side chain of R84 side chain of Ec-FhuD is shown by yellow highlight, corresponding coil (highlighted in yellow) in Sp-FhuD contains K106 with a possible projection towards binding cleft instead of R84 of Ec-FhuD.

3.7. Hydroxamate mediated radio labelled iron transport in pneumococci

It was difficult to find appropriate conditions in a minimal medium to determine the transport kinetics of [$^{55}Fe^{3+}$]ferrichrome and [$^{55}Fe^{3+}$]ferrioxamine B into *S. pneumoniae* R6. Therefore, transport was determined in THY broth supplemented with 0.4 mM nitrilotriacetate to reduce the available iron. Ferrichrome was transported into the wild-type strain R6, but not into the *ΔfhuD* mutant APD1, and was transported better into the *fhuD* complemented APD1C1 than into the wild-type (Fig. 38). In the latter case the *malM* promoter might be stronger than the *fhuD* promoter, thereby resulting in more FhuD and consequently more transport if the FhuD step is rate limiting. The transport of ferrichrome was twice as high as the transport rate of ferrioxamine B (Fig. 39)

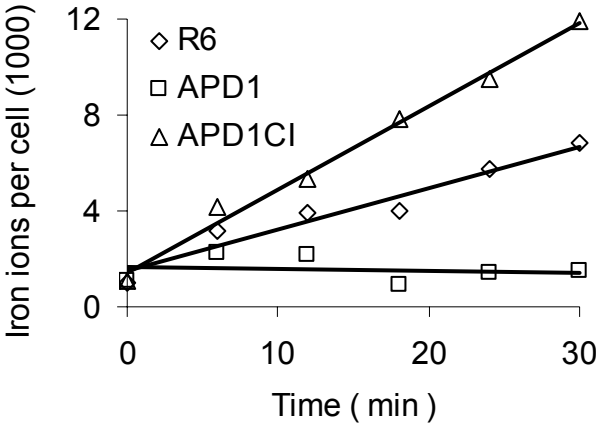


Figure 38. Radiolabelled ferrichrome mediated iron transport in *S. pneumoniae* R6 and mutants.

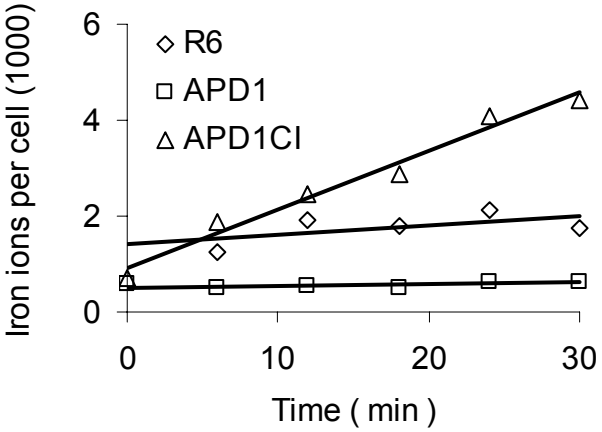


Figure 39. Radiolabelled ferrioxamine B mediated iron transport in *S. pneumoniae* R6 and mutants.

3.8. Streptonigrin toxicity assay determines ferric iron delivery by hydroxamates

The iron transport mutants were expected to show an increase in streptonigrin resistance since in *E. coli* sensitivity to streptonigrin depends on the intracellular iron concentration and has been used to isolate iron-supply mutants that are streptonigrin resistant (Braun *et al.*, 1983).

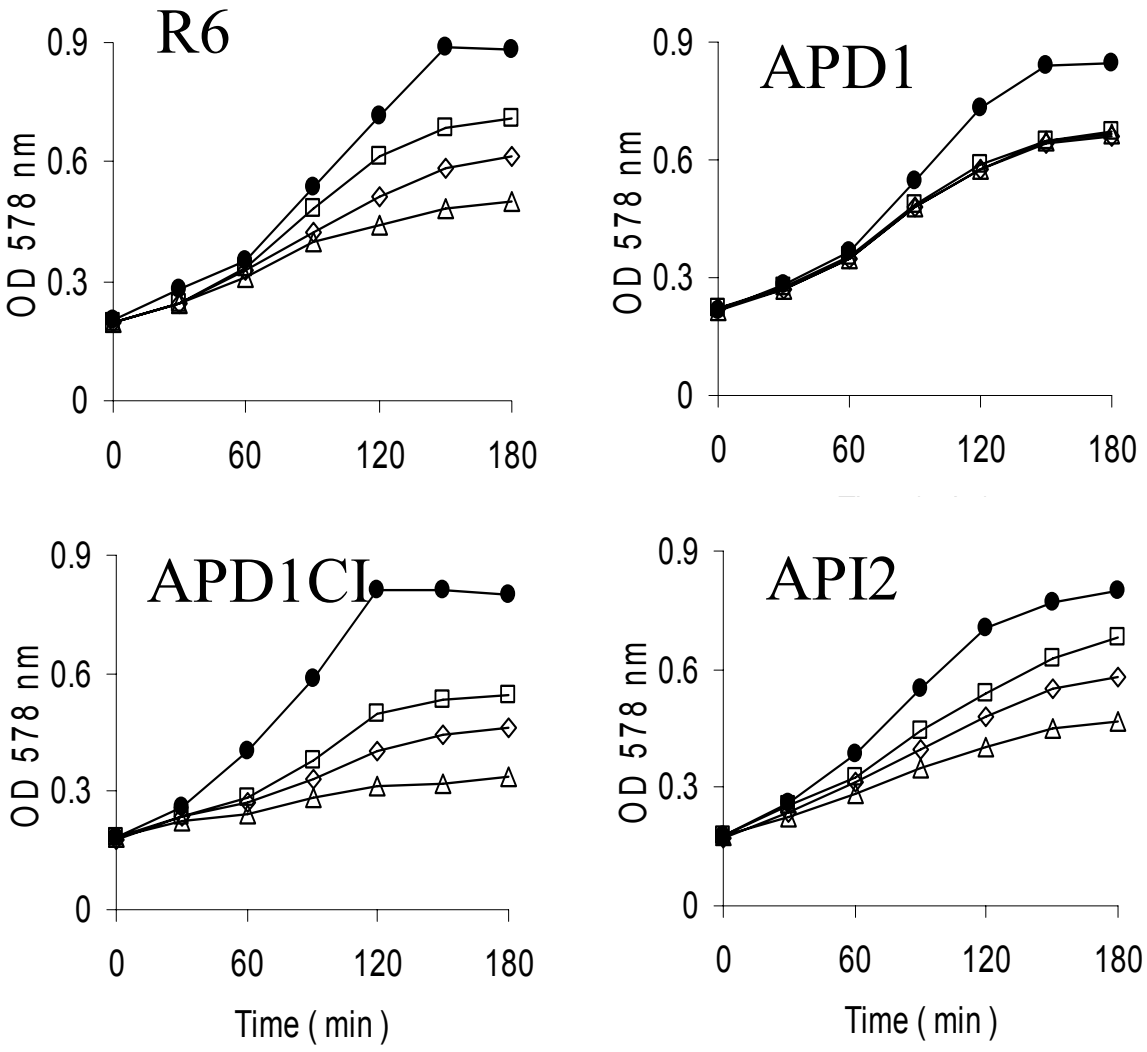


Figure 40. Sensitivity to streptonigrin (1 μg/ml) of *Streptococcus pneumoniae* R6 and the mutants APD1 *ΔfhuD*, APD1CI *fhuD*$^+$ of APD1, and API2 mutated in *spr1687*. Open squares, absence of ferrichrome and ferrioxamine B; open triangles, presence of 2.5 μM ferrichrome; open diamonds, presence of 2.5 μM ferrioxamine B; filled circles, no addition.

Cross application of filter papers soaked with ferrichrome and streptonigrin, respectively, on blood agar plates seeded with *S. pneumoniae* R6 resulted in a pronounced streptonigrin inhibition zone (Fig. 17). In liquid culture the streptonigrin sensitivity of mutant APD1 was not enhanced by ferrichrome and ferrioxamine B but sensitivity of the wild-type strain R6 and strain APD1CI was enhanced. The recombinant APD1CI was sensitive to albomycin and salmycin and showed enhanced sensitivity to streptonigrin in presence of ferrichrome and ferrioxamine B (Fig. 38, 39). Ferrichrome increased sensitivity to streptonigrin more strongly than ferrioxamine B that agrees with the higher ferrichrome transport rate as compared to the ferrioxamine transport rate.

3.9. Promoter analysis reveals a *fur* consensus sequence upstream of *fhuD*

Sequence analysis of the upstream promoter region of *fhuDBGC* operon revealed a fur regulator sequence (Fig. 41). The Fur-box overlapped –35 region and extended towards the –10 region of the promoter. A gram-positive ribosomal binding site (RBS) is placed exactly 5 nucleotides upstream of the methionine start codon of *fhuD*. The high conservation of consensus Fur-box sequence (17/19 match) was rather surprising in a gram positive lactic acid bacteria.

Consensus Fur-box GATAATGATAATCATTATC

fhuDBGC promoter -35 -10

AATTTGTAACTGTATCTA***TTGACA***ATGATAATTATTATCGA***TACAAT***AGACTTGAA

ATATGTTT**AAGGAG**TTTTT**ATG**

RBS Met-FhuD

Figure 41. Predicted promoter recognition sequence of the *fhuDBGC* operon. Fur-box homologous sequence (underlined) can be found between -35 to -10 region (bold italics) of the predicted promoter. Gram-positive ribosomal binding site (RBS) 5 bp upstream of the methionine start codon (bold face).

3.10. Inactivation of regulatory protein MarR and CiaR

The presence of a Fur-box prompted to look for the presence of *fur* homologues in pneumococcus genome. No close *fur* or *dtxR* homologue could be found in the

R6 and TIGR4 genomes. Scanning of R6 and TIGR4 genomes also did not reveal conserved Fur-box like sequences in addition to the *fhuDBGC* promoter. A tight regulation of iron and other metal ion uptake is expected from a peroxide producing but catalase negative species. Only a putative metal-dependent regulator *marR*, with sequence similarity to other metal dependent repressors in bacteria could be found upstream of the Mn transporter *psa* operon. From literature search on various microarray and global transcriptome data, another regulatory protein CiaR surfaced as a weakly probable regulator of *fhuDBGC* operon. Both *marR* and *ciaR* were independently inactivated. Neither API3 (*marR*$^-$) nor API6 (*ciaR*$^-$) showed considerable hypersensitivity to albomycin and salmycin. No clear conclusion regarding their role in regulation of the *fhu* operon transcription could be drawn from the results.

3.11. Purification of His-tagged MarR and recovery of bound DNA fragments by solid phase binding

Mutant API3 (*marR*$^-$) showed a 2-4 fold increase in albomycin sensitivity, but its poor growth compared to wild type and rapid decline in viability at stationary phase put caution on interpretation. The recombinant MarR (Spr1480) protein could be overexpressed in *E. coli* and purified without difficulty. It was expected that His_6-MarR would bind its recognition sequence. The chromosomal fragments containing a MarR recognition sequence will thus be enriched on the column with bound His_6-MarR. After eluting MarR-with bound DNA, the bound DNA can be recovered from the MarR-DNA complex under denaturing condition, cloned and sequenced.

His-tagged MarR was incubated with 1 μM each of Fe^{2+}, Mn^{2+} and Zn^{2+} as chloride salt in Tris-HCl, pH 6.8, then diluted 20 times and passed through a Ni-NTA agarose column to immobilize. Bound protein was washed thoroughly with Tris-HCl, pH 6.8 and then incubated with fragmented pneumococcal genomic DNA for 1 h, washed thoroughly with Tris-HCl buffer, pH 6.8, and the bound protein-DNA fragment was eluted with 250 mM imidazole. DNA was extracted from the eluent by phenol-chloroform extraction followed by ethanol precipitation. Purified DNA was further digested with a mix of blunt end producing restriction enzymes and cloned in SmaI digested pUC19. Few clones with insert were sequenced. None of the 10 sequenced clones contained regions nearby the *fhuDBGC* operon, putting doubt on the direct role of MarR on *fhuDBGC* operon transcription.

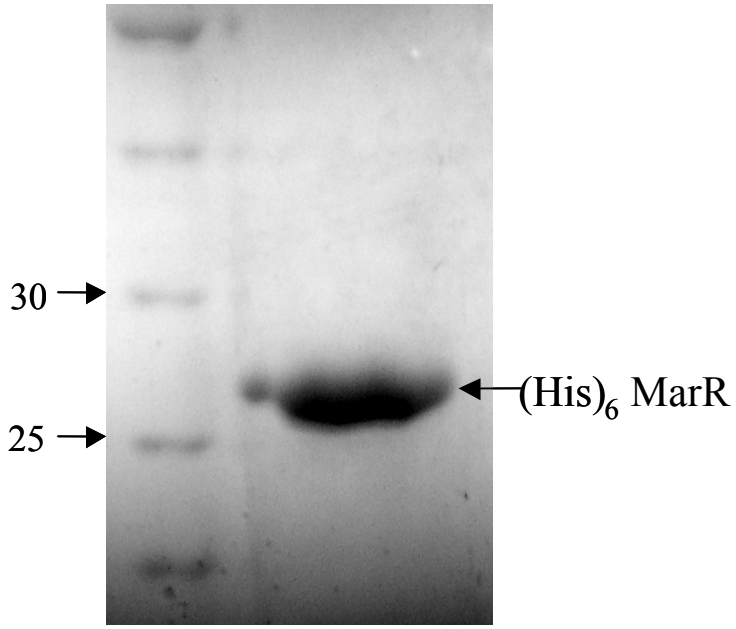


Figure 42. MarR was cloned and overexpressed in *E. coli*. His tagged MarR was purified by Ni-NTA affinity chromatography. His_6-MarR runs in SDS-PAGE as an ~ 26 kD protein band, when compared to protein molecular weight marker.

Table 13. Regions of chromosomal fragments recovered by solid phase DNA binding to His_6-MarR. Gene corresponding to actual recovered sequences are shown in bold face. The recovered fragments were cloned and sequenced.

Fragment 1		
	*spr*0027	hypothetical protein
***prs*A**	***spr*0028**	**Phosphoribosylpyrophosphate synthase**
	*spr*0029	Degenerate transposase (orf1)
Fragment 2		
*rr*08	*spr*0076	Response regulator
*hk*08	*spr*0077	Histidine kinase
***rps*D**	***spr*0078**	**30S Ribosomal protein S4**
	*spr*0079	Degenerative transposase
Fragment 3		
	*spr*0334	hypothetical protein
gnd	***spr*0335**	**6-phosphogluconate dehydrogenase**
*csr*R/*rit*R	*spr*0336	Response regulator
Fragment 4		
	*spr*0505	Phosphotransferase system sugar specific component
	***spr*0506**	**6phosphobetaglucosidase**
pheS	*spr*0507	Phenylalanyl tRNA synthetase alpha chain
Fragment 5		
	*spr*0612	Degenerate transposase
***pyr*F**	***spr*0613**	**Orotidine5'decarboxylase**
***pyr*E**	***spr*0614**	**Orotate phosphoribosyltransferase**
	*spr*0615	hypothetical protein

Fragment 6		
*xyl*H	*spr*0921	4oxalocrotonate tautomerase
tdk	***spr*0922**	**Thymidine kinase**
*blt*D	*spr*0923	Spermine/spermidine acetyltransferase
Fragment 7		
*tru*B	*spr*1092	tRNA pseudouridine 5S synthase
	***spr*1093**	**hypothetical protein**
	*spr*1094	hypothetical protein
Fragment 8		
*ala*S	*spr*1240	AlanyltRNA synthetase
	***spr*1241**	**hypothetical protein**
	*spr*1242	hypothetical protein
Fragment 9		
*rps*U	*spr*1271	30S Ribosomal protein S21
***nag*B**	***spr*1272**	**Nacetylglucosamine6phosphate isomerase**
*que*A	*spr*1273	S adenosylmethionine tRNA ribosyl transferase isomerase
Fragment 10		
*acp*S	*spr*1541	Acyl Carrier protein synthase
***aro*F**	***spr*1542**	**Phospho2dehydro3deoxyheptonate aldolase**
	*spr*1543	phospho2dehydro3deoxyheptonate aldolase

3.12. Inactivation of the ferrochelatase and the pyruvate oxidase

Working on pneumococcal iron transport faced the difficulty of the lack of a suitable iron depleted medium. It has been observed that pneumococci could grow well in complex liquid broth in presence of up to 1 mM NTA or Dipyridyl or EDDHA. Many attempts were made to standardize a chemically defined medium with iron depletion to observe a reversible growth arrest of pneumococci. Only very few chemically defined medium recipes can be found in the literature. Depletion of iron with specific chelators like EDDHA or dipyridyl in such chemically defined medium was not sufficient to restrict pneumococcal growth. The chemically defined medium contained 20 amino acids, few vitamins, readily available carbohydrates and was not likely to put much pressure under in vitro conditions to bacteria with no cytochrome-Krebs cycle. To get a better understanding for developing an iron restrictive chemically defined medium for pneumococci, pyruvate oxidase (*spxB*) and the putative ferrochelatase gene were independently inactivated. Till now by modification and trials of existing chemically defined medium one recipe was formulated as presented in materials and methods. This medium presents some positive responsiveness in terms of pneumococcal growth but results showed poor reproducibility. Further characterization of that medium is necessary.

3.13. Albomycin activity *in vivo*

Albomycin was found to be highly active against a broad spectrum of gram negative and gram positive pathogens in vitro. At this point it was interesting to address how albomycin works in an in vivo infection model. Activity of albomycin was checked separately against a gram positive and a gram negative species in a murine infection model.

3.13.1. Reduction of *Yersinia* load in spleen with albomycin in mice

Yersinia enterocolitica (Table 9) is sensitive to albomycin in vitro. Earlier it was reported that the outer membrane protein FcuA in *Y. enterocolitica* facilitates uptake of ferrichrome (Baumler and Hantke, 1992; Koebnik *et al.*, 1993; Stojiljkovic *et al.*, 1994). For the study of the efficacy of albomycin against *Y. enterocolitica* in a murine model, virulent strain 8081 of serotype O8 was selected.

Mice were infected with *Y. enterocolitica* for 24 h. Placebo (PBS) or antibiotics (control; gentamycin, test; albomycin) at the rate of 10 mg / kg body weight were injected 24 h post-infection. Bacterial load in spleens were recorded 6 h and 24 h after the single dose treatments. In each group there were 6 mice. Gentamycin is the choice of treatment against yersiniosis in humans, and was chosen as control treatment. After 6 h of treatment albomycin reduced the bacterial load even better than the identical amount of gentamycin, but was found to be less effective after 24 h post treatment. In gentamycin treated mice spleen CFU is still going down, whereas in albomycin treated mice infection started to recover, though was still ~ 2 log lower CFU than in the placebo treated mice spleen. This could be due to, lower absorption in animal tissue and thus quicker extinction from circulation. To check that three healthy mice were injected with identical dose of albomycin and blood samples were collected from tail vein at time intervals of 5, 15, 30, 60, 120, 240 min. Blood samples were diluted two fold with PBS, and their inhibitory effect on *Y. enterocolitica* 8081 was checked. Double diluted blood samples collected after 60-120 min of albomycin injection did not show any inhibitory effect in vitro, though similarly diluted samples collected before that time point showed visible inhibition zone on plate.

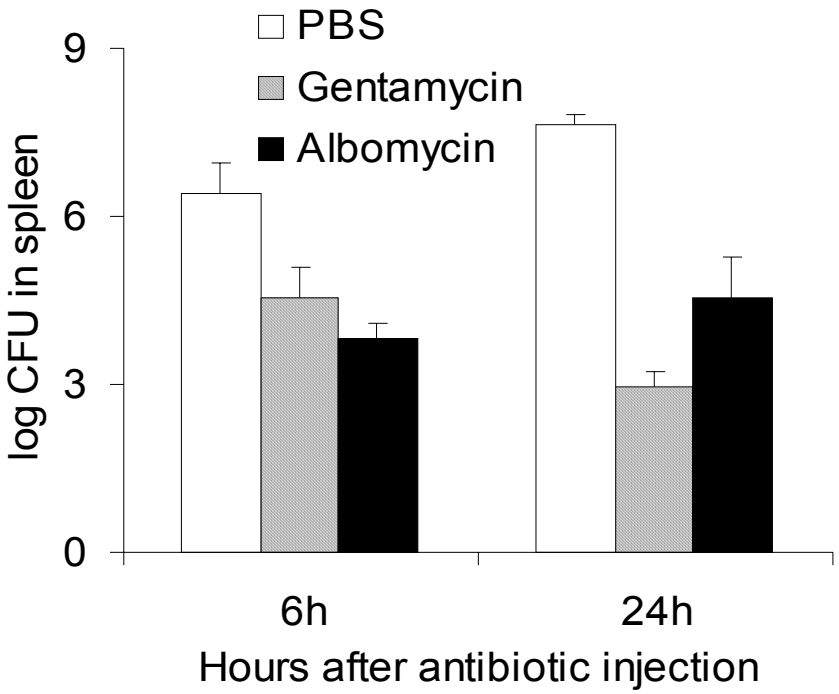


Figure 43. Mice were infected with $5x10^4$ CFU of *Y. enterocolitica* strain 8081. The infection was allowed to proceed for 24 h prior to treatment with a single dose of either albomycin or gentamicin and subsequently sacrificed 6 h and 24 h post treatment. The spleen removed and the number of bacteria enumerated. Each column represents mean of the counts from 6 mice in the treatment group, bar represents the standard deviation.

3.13.2. Competitive index of spontaneous *Yersinia* mutants arising in vivo

Development of spontaneous albomycin resistance occurs with a frequency of ~1 in 10^4- 10^5 (variations observed with inoculum age and growth conditions) for *Y. enterocolitica* when grown and tested in vitro. The competitive index (CI) is defined as the change in the ratio of two strains after growth together under the same experimental conditions (Freter *et al.*, 1981; Taylor *et al.*, 1987). By definition CI for a defined mutant is calculated by dividing the output ratio (mutant / wild type) by the input ratio (mutant / wild type), where a competitive index of 1 indicates that the two strains are proliferating equally in vivo and less than 1 indicates relative attenuation of the respective mutant against wild type.

Spontaneous albomycin resistance development can be caused by independent mutations in multiple genes. To study efficacy of the test antibiotic and development of spontaneous resistant mutants in vivo, the CI calculation is modified to calculate CI_{mutant}, defined as the change in output ratio (mutant CFU / total CFU) from the input ratio (spontaneous mutant / total CFU). Where a CI_{mutant} value, 1 indicates that mutants are

proliferating in vivo equally well as the wild type and less than 1 indicates relative attenuation of the mutant against wild type. In this calculation upper limit of CI_{mutant} would be reached when recovered CFU is only mutant and no wild type (as is expected under antibiotic selection pressure where wild type is sensitive to the test antibiotic). Thus for an effective test antibiotic, the total recovered CFU should decline sharply after the start of treatment, but the CI_{mutant} would be reaching closest to upper boundary. CI_{mutant} in absence of antibiotic will indicate the comparative virulence of mutants with respect to wild type.

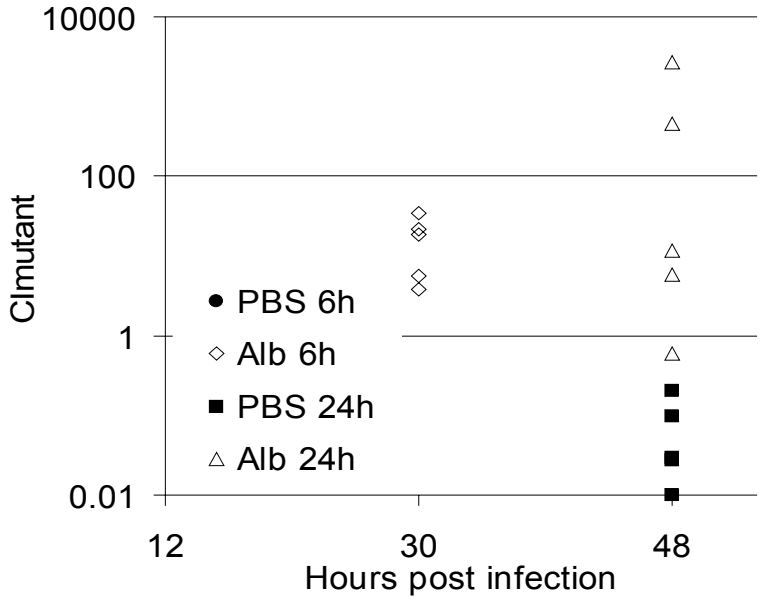


Figure 44. CI_{mutant} of albomycin resistant *Y. enterocolitica* mutants in the murine infection model with single dose of albomycin was plotted. The mice were infected with $5x10^4$ CFU of *Y. enterocolitica* 8081 containing an average of 5 spontaneous albomycin resistant mutants. Mice were subsequently treated with either, albomycin (10 mg/ml) or PBS at 6 h and 24 h post infection. Mice were then sacrificed 30 h and 48 h post infection, the spleen was removed and the number of albomycin resistant and total bacteria enumerated. At the 30 h time point no albomycin resistant mutants were isolated in the PBS only group. Each point in the plot represents CI_{mutant} calculated from individual infected mice spleen, with 5 mice in each group.

At each time point (30 h and 48 h post infection) CFU of total bacterial load and albomycin resistant were scored from spleen of each experimentally infected mouse separately, CI_{mutant} was plotted on a log scale. In the absence of albomycin, 30 h post infection spontaneous albomycin resistant CFU in spleen were below detectable limit, thus no CI_{mutant}

could be calculated. Detectable albomycin resistant mutant could be found by 48 h post infection and was still considerably lower than CI_{mutant} value of 1, indicating attenuated virulence of resistants. On the other hand after single albomycin injection, 30 h post infection total CFU declined > 2 log and CI_{mutant} of treated mice went higher than 1, confirming effective clearing of sensitive population by albomycin.

3.13.3. Recovery from experimental pneumococcal infection in mice by use of albomycin

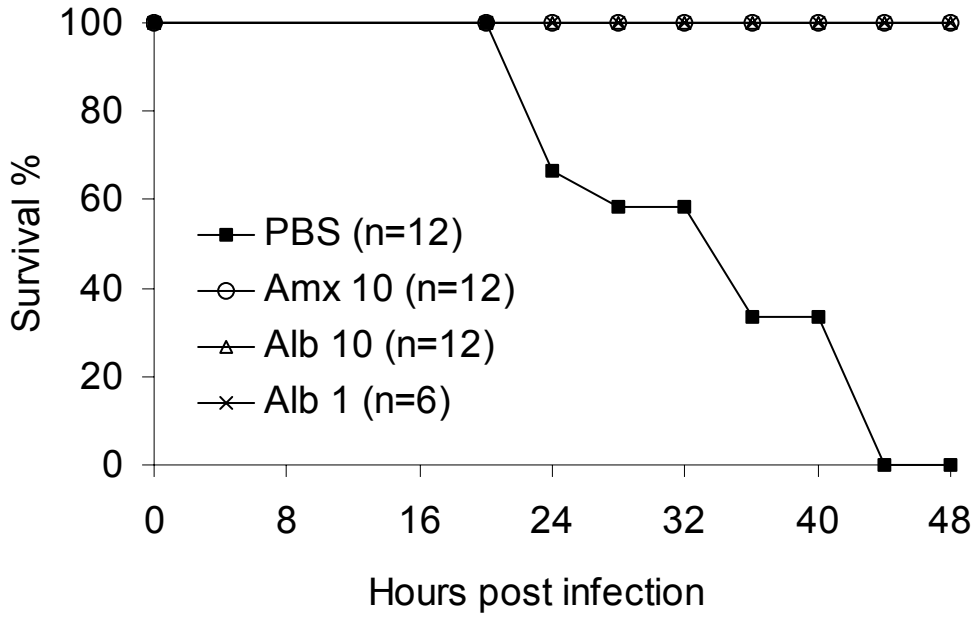


Figure 45. Survival of mice after albomycin treatment to recover from experimentally induced pneumonia. Mice were experimentally infected with D39 and antibiotics were used in four treatment groups; 1. placebo (PBS, n=12), 2. albomycin 1 mg / kg / dose thrice a day for 2 days (Alb1, n=6), 3. albomycin 10 mg / kg / dose thrice a day for 2 days (Alb10, n=6), 4. amoxicillin 10 mg / kg / dose thrice a day for 2 days (Amx10, n=6). Survival was monitored regularly and death at hours post infection was recorded.

Efficacy of albomycin in vivo was also checked against a gram-positive organism in mice. In the earlier part of the study, *S. pneumoniae* was found to be sensitive to albomycin in vitro and the transport system was characterized. Thus in vivo studies extended with the virulent D39 strain of pneumococcus and an isogenic albomycin resistant strain D39T1. Treatment in all the groups started 12 h post bacterial infection. All placebo treated mice died within 48 h post infection while amoxicillin (10 mg /kg) and 6 doses albomycin

both at a high (10 mg/ kg /dose) or as low as 1mg /kg every 8 h interval rescued 100% mice from the infection. Survival and recurrence of infection of the treated mice were monitored till 2 weeks after the last dose of antibiotic.

3.13.4. Albomycin resistant pneumococci are less competitive in vivo

Competitiveness of the albomycin resistant mutants in vivo was studied using coinfection of parent D39 and an isogenic albomycin resistant strain D39T1. Mice (n= 12) were infected with a mix of 1000 CFU of D39 and 14 CFU of D39T1. After 12 h post infection two treatment groups with 6 mice in each were made, one group received placebo (PBS) and the other group received 6 doses of albomycin (10 mg / kg / dose, thrice a day). In 8 h intervals blood samples were collected from the tail vein of each mouse for bacterial counts. All PBS treated mice died within 26 h post infection. Albomycin treated mice were alive till 56 h, after that they were sacrificed due to ethical reasons.

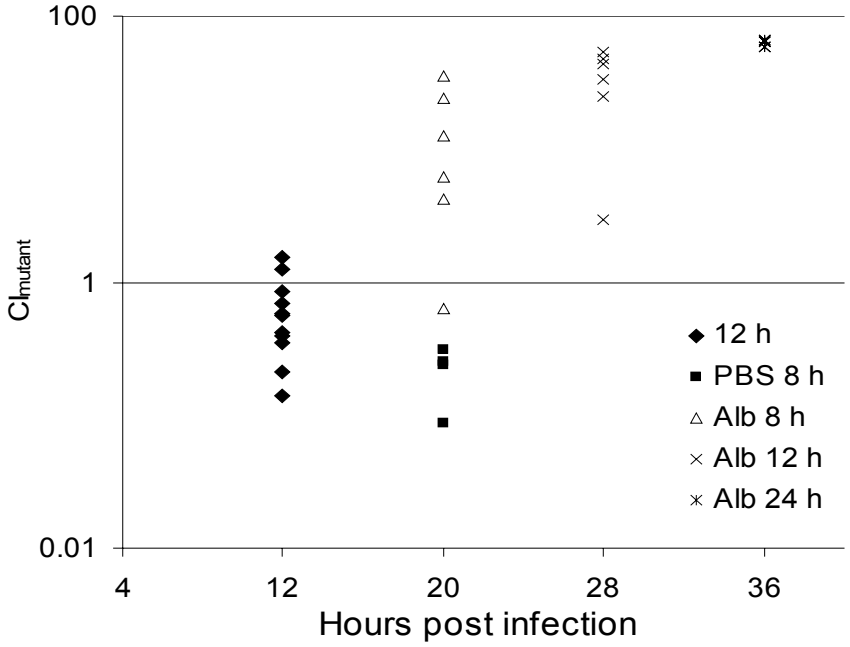


Figure 46. CI_{mutant} of albomycin resistant *S. pneumoniae* D39T1 mutants in the murine infection model with multiple doses of albomycin was plotted. The mice were infected with a mixture of wild type *S. pneumoniae* D39 and an isogenic albomycin resistant mutant *S. pneumoniae* D39T1 at a ratio of approximately 70:1. Mice were treated with either, albomycin (10 mg/ml) or PBS at 12 h, 20 h or 28 h post infection. The blood of the mice was sampled at 12 h, 20 h, 28 h and 36 h post infection and the number of albomycin and wild

type bacteria enumerated. Each point in the plot represents CI_{mutant} calculated from individual infected mice, with 6 mice in each group.

CI_{mutant} of albomycin resistant mutants before the start of treatment within 12 h post infection i.e. in infection establishment period, were mostly below 1, indicating a moderate disadvantage of the mutants in infection establishment. By 20 h post infection without albomycin selection CI_{mutant} continued dropping further below 1, indicating severe disadvantage of the resistant mutants in infection progression. On the other hand when the co-infected mice were treated with albomycin the total count recovered from blood decreased while CI_{mutant} gradually went higher than 1 and reached the upper boundary limit (1000/14 = 71.5) within 24 h of the treatment start, meaning effective eradication of sensitive population. This result provided evidence for a very high efficacy of albomycin in vivo against pneumococci.

3.14. Development of a pneumococcal cloning plasmid

Plasmid borne cloning in pneumococcus was not found to be very common in literature, except for plasmid pMV158 and its derivatives. Unlike most other common theta replicating plasmids used for cloning, pMV158 replicates by a rolling circle mechanism and needs *recA*$^+$ *polA*$^+$ host. The pMV158 derivative that could be modified for the study was pLS101, a derivative of pMV158 containing the maltose utilization genes from *S. pneumoniae*. Multiple trials to clone large *fhu* transport fragment in this vector was not fruitful. Successful cloning of *fhu* in pLS101 would lead to a plasmid containing two fragments (*mal* and *fhu*) that have homologues in the chromosome. Expecting that might reduce the chance of successful cloning of *fhu* operon, a derivative of pLS101 without any chromosomal homologues region but containing an easy multiple cloning sites was desired. To achieve this goal pLS101 was digested with HindIII - PstI, a large fragment containing the tetracycline resistance marker and part of the replication origin was fused into pJDC9 to produce pTAP1. This plasmid replicates in *E. coli* but not in pneumococci by the theta mode. The pMB9 replication origin in pJDC9 and the pMV158 replication origins in pLS101 were found to be incompatible since they made the fusion plasmid unstable. The rest of the pLS101 replication origin was cut out by PstI digestion and cloned into PstI digested pTAP1. The resultant construct could not be cloned directly into either pneumococcus or *E. coli* with both the replication origins present. This construct was digested with HaeII and religated to deactivate pMB9 replication origin. Transformation in pneumococci resulted in plasmid

pRCAP1 with both erythromycin and tetracycline resistance markers. pRCAP1 also shares pUC19 multiple cloning site, though there were 3 EcoRI site, distributed throughout the plasmid. Partial EcoRI digestion and re-ligation produced pRCAP2 releasing *ermB* and most of the pJDC9 derived part resulting in a manageable sized (~ 4.5 kb) plasmid for cloning into pneumococcus. Though this plasmid was found to be efficient in cloning up to at least 2 kb of random pneumococcal fragment into *S. pneumoniae* R6, attempts to use this plasmid in cloning Sp-*fhuDBGC* in pneumococci failed.

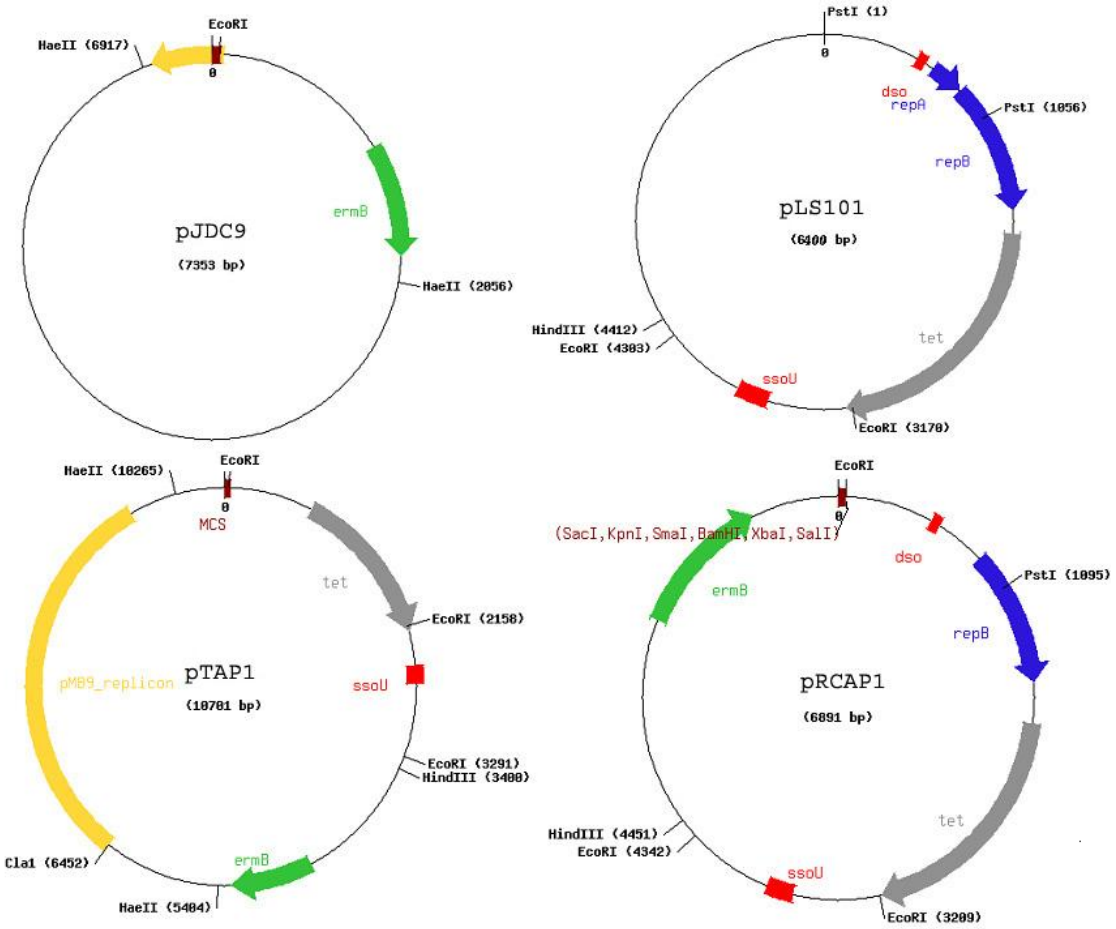


Figure 47. Line drawing of plasmids pJDC9, pLS101, pTAP1 and pRCAP1 showing resistance marker genes, relevant restriction enzyme recognition sites and replication origins. dso; double strand replication origin, ssoU; single strand replication origin (both dso and sso is necessary for rolling circle replication), *ermB*; erythromycin resistance marker, *tet*; tetracycline resistance marker, MCS (multiple cloning site) with unique enzymes.

4. Discussion

Balancing the iron metabolism is quite an Achilles heel for virtually all living organisms. Iron is much of an indispensable element for carrying out redox reactions in respiration and many other known and unknown vital cellular processes. Bacteria and all other living organisms deservingly spend much effort and energy to acquire balanced diet of iron on a continuous basis. To meet the need of iron continually in ever changing competitive environments, successful bacteria employ multiple strategies from synthesizing their own siderophores or to acquire iron loaded chelators from other organisms, even at the cost of large genetic burden to carry (Braun and Killmann, 1999; Braun, 2003; Ganzoni and Puschmann, 1975; Green and Paget, 2004; Hoen, 1999; Neilands, 1973; Neilands and Nakamura, 1985; Neilands, 1995). In well studied *E. coli* nearly 1 % of total genetic material is devoted to multiple iron import systems. Though all the transport systems do not serve the host equally well at any given environmental condition (Skaar *et al.*, 2004) and mutations at those transport systems occur frequently with dispensible effect on vitality in complex environment. Surprisingly, shedding of iron transport genes from a number of available iron acquisition systems is anything but least common among respiratory bacteria; rather bacterial pathogenicity island acquired by horizontal transfer often carry a surplus iron transport systems (Anisimov *et al.*, 2005; Brown *et al.*, 2004; Hare *et al.*, 1999; Janakiraman and Slauch, 2000; Koczura and Kaznowski, 2003; Kunkle and Schmitt, 2003; Luck *et al.*, 2001; Mey *et al.*, 2005; Mokracka *et al.*, 2003; Moss *et al.*, 1999; Schubert *et al.*, 1998; Schubert *et al.*, 2002; Sorsa *et al.*, 2003; Zhou *et al.*, 1999). This shows how a slight edge provided by a specific transport system could be turned into a big advantage for a species. Permanent disposition of any iron-substrate transport system is likely to be unfavourable for any bacterial species. Sideromycins are a natural clue how to exploit this window by Trojan horse approach to deliver antibacterials aiming to clear up undesired bacterial population. Some known sideromycins are ferrimycin, danomycin, salmycin and albomycin (Bickel *et al.*, 1965; Braun *et al.*, 1983; Fujii *et al.*, 1964; Gause, 1955; Miller *et al.*, 1991; Neilands, 1976; Roosenberg and Miller, 2000; Sackmann *et al.*, 1962; Stapley and Ormond, 1957; Tsukiura *et al.*, 1964; Vorisek and Grunberger, 1966; Yamada and Kawaguchi, 1964).

4.1 Overproduction of sideromycins

Though sideromycins like albomycin are potentially attractive candidates for pharmaceutical attention, the biggest problem lies in cost effective production. Approaches to identify biosynthetic genes cluster are underway to help increase the albomycin production (unpublished V. Braun). For the present study variations of available fermentation conditions were evaluated in terms of albomycin yield from fermentation broth (Fiedler, 1985). It was found that all the albomycin producing strains are not equally good for albomycin production. Moreover within a strain, after sporulation different spore lineage produce different levels (0 - 40 mg/l) of albomycin under similar growth condition. Better aeration was always accompanied with better production. Ornithine is a direct building block of albomycin, thus the positive correlation of albomycin production with the addition of ornithine is expected to have a direct effect of building block availability. Another striking finding is that elevated albomycin production can be triggered by addition of excess iron (1 mM) after growing streptomycetes to late log phase under moderate iron starvation. While no albomycin could be detected when grown under iron starvation the fermentation broth contains higher levels of unsaturated ferrioxamine species. This would be due to deregulation of siderophore synthesis for acquisition of iron under iron scarcity. When this culture was supplemented with 1 mM the broth turned red indicating saturation of the ferrioxamines. The ferrioxamines would start supplying the iron-derepressed cells with a sudden flux of iron. Under such iron sufficient conditions, albomycin production is triggered which leads to a maximum concentration in the fermentation broth by 24 h and maintains the level for another 72 h. This provided a big window of time to harvest for further purification. Many streptomycetes are known to produce and to be able to utilize hydroxamates as iron substrates (Fiedler *et al.*, 2001; Meiwes *et al.*, 1990; Muller and Raymond, 1984; Rivier *et al.*, 1983; Schupp *et al.*, 1988; Yamanaka *et al.*, 2005; Yang and Leong, 1982). It could be a good strategy for hydroxamate producing streptomycetes to secrete structurally related sideromycins under iron sufficient conditions to restrict quick growing competitive species either by creating a pseudo iron stress, or by delivering the antibiotic through the analogous siderophore specific transport system.

4.2 Wide distribution of hydroxamate transport system

Activity of albomycin has long been used as an indicator of functional ferrichrome transport systems, especially in *E. coli* (Braun *et al.*, 1991; Dolence *et al.*, 1991;

Ferguson *et al.*, 2000; Fischer *et al.*, 1989; Killmann and Braun, 1992; Koster and Bohm, 1992; Mademidis and Koster, 1998; Rohrbach *et al.*, 1995; Schneider and Hantke, 1993). The iron chelating part of albomycin is directly analogous to ferrichrome (Fig. 3). Moreover despite differences in structure the hexadentate iron coordination in other hydroxamates (ferrioxamines and salmycin) are also very similar. This part of the structures may form the major recognition site for the transport- binding proteins, as was determined in the crystal structures of the *E. coli* FhuA and FhuD proteins loaded with albomycin, ferrichrome, and ferrioxamine B (Braun, 1999; Clarke *et al.*, 2000; Clarke *et al.*, 2002; Ferguson *et al.*, 1998; Ferguson *et al.*, 2000; Ferguson and Deisenhofer, 2002; Krewulak *et al.*, 2005; Turkova *et al.*, 1963). Though both FhuA and FhuD interact with hydroxamates with more or less discrimination, the mechanism is very different. FhuA senses the high affinity docking of the substrate (ferrichrome and albomycin) and let the substrate to slide through the entire length of the protein to reach periplasmic space. Covering the whole substrate by FhuA allows to discriminate the structural variations posed by different hydroxamates like ferrichrome (transported) and ferrioxamine B (not transported). This is not the case with FhuD. The structure of *E. coli* FhuD with loaded substrate has been resolved at the atomic level and reveals recognition of the ferric hydroxamate centre. Distinct structures are observed in substrate-loaded FhuD proteins as compared with unloaded FhuD. Molecular dynamic simulations of the *E. coli* FhuD (Clarke *et al.*, 2001; Krewulak *et al.*, 2005) and small angle crystal scattering of the *S. aureus* FhuD (Sebulsky *et al.*, 2003) revealed small changes upon substrate binding. The forceps like holding of hydroxamate type iron coordination centre leaving other parts of the substrate free by FhuD might allow wider substrate binding than FhuA.

When the activity spectrum of albomycin was scanned against a broad array of common bacterial pathogens, the ferrichrome transport system was found to be widely distributed. This was a striking observation as ferrichrome is known to be synthesised by phytopathogenic fungi *Ustilago* and is less likely to be encountered inside a human host (Budde and Leong, 1989; Ecker *et al.*, 1982; Emery, 1971; Wang *et al.*, 1989). The present speculation is that compounds that coordinate iron similarly to the way hydroxamates coordinate iron might be present and recognized by the bacterial hydroxamate transport systems. Most of the enterobacteriaceae except *Proteus-Morganella-Providencia* (PMP) group was found to be ferrichrome positive, since they are albomycin sensitive. Whereas most frequently encountered organisms in cystic fibrosis (*Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*) were non-responsive to albomycin, except

Alcaligenes xylosoxidans which showed sensitivity under iron limited conditions. Among the gram positives, *S. aureus* and *S. pneumoniae* were highly sensitive to albomycin.

4.3 Siderophore dependent iron acquisition by pneumococcus

In *S. pneumoniae* R6, genes *spr0934-0936*, and *spr0938* encoded a ferric hydroxamate transport system through which ferrichrome, ferrioxamine B, albomycin and salmycin were taken up into cells. Ferrichrome and ferrioxamine B interfered with the activity of the antibiotics. The two antibiotics inhibit protein synthesis - albomycin interferes with serine loading of the seryl-tRNA, and salmycin inhibits at an unknown target (V. Braun, unpublished results). Therefore, the ferric hydroxamates did not inhibit at the antibiotics target sites but interfered with their transport. This conclusion is supported by the phenotype of the transport negative mutants in the ferric hydroxamate transport genes that were resistant to the antibiotics. The definite antibiotic resistance phenotype of the mutants indicates a single ferric hydroxamate transport system. This finding is supported by the low sequence identity between the *fhu* genes and the related genes of the two other putative iron transport systems of *S. pneumoniae* R6, which range from 10% to 32%.

Although the iron transport rates by the hydroxamates in *S. pneumoniae* was low - less than 10% of the transport rates with *E. coli* - the rate was sufficient to render cells highly sensitive to albomycin. The concentration of albomycin that inhibited the synthetase was comparable to the MIC. The low transport rate in *S. pneumoniae* might be caused by a sufficient iron supply in the rich medium in which the bacteria were grown. The amount of nitrilotriacetate added to the medium to complex the iron was probably not sufficient to reduce the iron level to a growth-limiting concentration. The situation is further compounded by the probable low iron requirement of *S. pneumoniae* which does not contain membrane-bound electron transport chains or have a TCA cycle in which most of the iron of respiratory bacteria is used.

The genes involved in ferric hydroxamate transport in *S. pneumoniae* R6 were designated as *fhuD* (encodes binding lipoprotein), *fhuB* and *fhuG* (encode transmembrane transport proteins) and *fhuC* (encodes ATPase). This nomenclature agrees with that of *Bacillus subtilis* from which the first ferric hydroxamate transport system of gram-positive bacteria was partially characterized (Schneider and Hantke, 1993), *Staphylococcus aureus* (Sebulsky *et al.*, 2003) and a B group streptococcus (Clancy *et al.*, 2006). The *fhuD fhuB fhuG fhuC* genes of *S. pneumoniae* are transcribed in the same direction and most likely form

an operon. *B. subtilis* has the same gene order but *fhuD* is transcribed in the opposite direction (Kunst *et al.*, 1997; Schneider and Hantke, 1993). In *S. aureus* the gene order is *fhuC fhuB fhuG* whereas *fhuD1* and *fhuD2* are located at other sites on the chromosome (Sebulsky and Heinrichs, 2001). In the group B streptococcus all four genes have the same transcription polarity but are arranged *fhuC fhuD fhuB fhuG* (Clancy *et al.*, 2006). An iron transport system was studied in a clinical isolate of *S. pneumoniae* 0100993. This system was first designated *pit2* (Brown *et al.*, 2001), and then *pia* (Brown *et al.*, 2002) since the transport substrate was not identified. $^{55}Fe^{3+}Cl_3$ uptake is not lower in a *pit2A* mutant (*fhuD*) and requires a second mutation in *pit1B* (*spr*1684 of Fig. 18) to be 73 % lower than that of the wild-type after 15 min to 30 min incubation (Brown *et al.*, 2001). Growth of the *pit2A* mutant is more strongly reduced than that of the wild type in THY medium treated with Chelex-100 to remove iron and is restored by addition of $FeCl_2$. Sensitivity of the *pit2A* mutant to streptonigrin is reduced. Unfortunately, a nomenclature other than *fhu* was also used in a recent study of a ferrichrome uptake system in *Streptococcus pyogenes* (Hanks *et al.*, 2005). The *ftsA ftsB ftsC ftsD* genes, as organized on the chromosome, correspond to the *fhuC*, *fhuD*, *fhuB* and *fhuG* genes, respectively. The clinical *S. pyogenes* strains we examined were resistant to albomycin and salmycin. Either the described Fts system is much more specific than the hitherto studied ferrichrome transport systems, or the antibiotic moieties are not released from the iron carriers. Heme was shown to be another iron source for *S. pneumoniae* (Tai *et al.*, 1993). PiuA encoded by *spr1687* bound to haemin-agarose and more weakly to haemoglobin-agarose and isolated PiuA bound heme (Tai *et al.*, 2003).

Surprisingly streptococcal species other than pneumococci were not sensitive to albomycin, and salmycin is active against pneumococci and GBS (*S. agalactiae)* but not against GAS (*S. pyogenes*) isolates. Such nonlinearity of the distribution of ferric hydroxamate transport system among streptococcal species could only arise if the *fhuDBGC* operon in pneumococci is acquired horizontally after it separated from other streptococcal species. Indeed the *fhuDBGC* loci resides in the 27 kb pathogenicity island PPI-1 (Brown *et al.*, 2001). Though all the *S. pneumoniae* strains covering 10 capsular types tested till date are highly conserved for *fhuDBGC*, the PPI-1 contains 28 other genes in the strain TIGR4 and shows considerable variation of gene content from strain to strain (Brown *et al.*, 2004; Whalan *et al.*, 2006). The high conservation among pneumococcal isolates and the antigenicity of *fhuD* has shown promise as potential vaccine candidate (Brown *et al.*, 2001; Jomaa *et al.*, 2005; Whalan *et al.*, 2005). In the *S. pneumoniae* R6 genome 40 open reading frames are predicted to be derived from gram-negative bacteria, and this might be a

consequence of competence (Hoskins *et al.*, 2001). The finding that the putative iron transport system encoded by the s*pr0224*/*0223* is disrupted agrees with the observation that many open reading frames for transporters are disrupted in *S. pneumoniae* R6 and suggests that truncated foreign genes were acquired, or *S. pneumoniae* genes not required for growth in the fastidious *S. pneumoniae* environment were mutated (Bruckner *et al.*, 2004; Claverys *et al.*, 2000; Hakenbeck *et al.*, 2001; Havarstein *et al.*, 1997; Hollingshead *et al.*, 2000).

Until recently mechanism of iron uptake by gram-positive pathogens remained mostly unattended. A flurry of reports in the post genomic years brought better insights into the gram-positive iron transport systems. Most prevalent mechanism of reported iron uptake employed by gram-positive species as presented in Fig. 2. The simplest model of iron transport directly relies on four components, a membrane bound lipoprotein captures iron-substrate from environment and presents it to the transmembrane permease complex formed with two heterodimeric oligopeptides energized by two copies of an associated ATP hydrolysing protein. Involvement of multiple cell wall anchored binding proteins in addition to a membrane anchored lipoprotein for heme passage in *S. aureus* is reported recently (Marraffini *et al.*, 2006; Mazmanian *et al.*, 2003), where typical membrane spanning permease components and a cognate ATPase reside elsewhere in the chromosome. Similar observation was made in *S. aureus* for hydroxamate transport (*fhuCBG*) and staphylobactin transport (*sirABC*) where both use *fhuC* as the cognate ATPase (Speziali *et al.*, 2006).

Unrooted dendrogram based on sequence alignment of pneumococcal *fhuD* with the hydroxamate binding proteins from *Bacillus subtilis*, *Staphylococcus aureus* and *E. coli* strengthened the assumption of horizontal transfer of the *fhuDBGC* from a gram negative species (Fig. 48). In essence transport specificity of the iron transport in gram-positive bacteria rely mostly on substrate binding to the lipoprotein. It is tempting to assume that, as long as the substrate binding protein is able to cross talk with a cognate permease-ATPase complex it can send its substrate inside the cell. Thus gene duplication followed by accumulation of mutations in substrate binding pocket altering substrate binding specificity can allow a species to access variety of nutrients. Indirect evidences of such strategy reported in *S. aureus* and *B. subtilis*, where two hydroxamate binding proteins with varying substrate specificity arose from possible gene duplication to utilize different ferric hydroxamates substrates in concert with a common permease – ATPase complex (Schneider and Hantke, 1993; Sebulsky *et al.*, 2004).

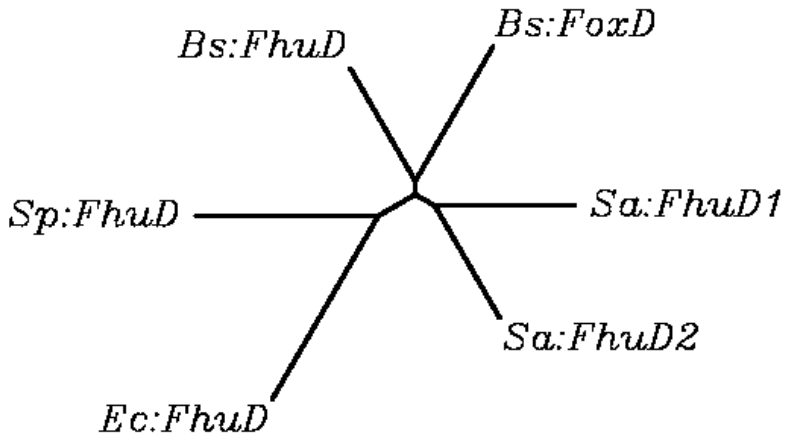


Figure 48. Unrooted dendrogram of hydroxamate binding proteins from *S. pneumoniae* (Sp), *Bacillus subtilis* (Bs), *Staphylococcus aureus* (Sa) and *E. coli* (Ec) based on sequence alignment.

The genera *Lactobacillus, Leuconostoc, Pediococcus and Streptococcus* are classified as lactic acid bacteria. Food fermented with *Lactobacillus* and *Leuconostoc* make an important part of the human diet, while streptococcal species can diet on living beings. In the pre genomic era the role of iron in the metabolism of *Lactobacillus* and *Streptococcus* was highly doubted (Archibald, 1986; Posey and Gherardini, 2000). Lack of evidence for the presence of cytochromes, TCA cycle enzymes, siderophore production & transport, azide inhibition and moreover catalase independent high peroxide tolerance with Mn-dependent SOD has lead to the common assumption of an iron independent metabolism in lactic acid bacteria (Bruno-Barcena *et al.*, 2004; Deibel and Evans, 1960; Efthymiou and Joseph, 1974; Gaillot *et al.*, 1997; Jakubovics and Jenkinson, 2001; Jakubovics *et al.*, 2002; Jurtshuk and McQuitty, 1976; Martin *et al.*, 1984; Massa *et al.*, 2001; Poyart *et al.*, 2001; Ramadan, 1968; Reuter, 1992; Sato, 1972; Smith and Bodily, 1967; Yoshpe-Purer, 1989). The availability of genome sequences of multiple species of *Streptococcus* and *Lactobacillus* suggested a rather unprecedented scenario. Though homologues genes for siderophore production or complete TCA cycle could not be found, multiple putative iron transporters, anaerobic type cytochromes, ferridoxin, and few heme biosynthetic enzymes were prevalent (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). Such ambiguities lead to a recent surge of attention towards the iron transport systems among streptococcal species. Lack of suitable iron limited media for streptococcal growth assays, restricted most findings on the presence of a iron transport system without detailed characterization of substrate specificity (Brown *et al.*, 2001; Brown *et al.*, 2002; Tai *et al.*, 1993; Tai *et al.*, 2003). In this study, use of albomycin and salmycin lead

to the identification of a siderophore (hydroxamate) specific iron transport system in pneumococcus.

4.4 Non-transferable resistance to albomycin

Many gram-negative and few gram-positive species are albomycin sensitive and all the sensitive bacteria take up albomycin via ferrichrome transport systems. Species of *Proteus* were intrinsically resistant to albomycin. The resistance is due to lack of ferrichrome uptake machinery and not due to presence of an albomycin detoxifying or efflux system. Such resistance mechanism is not transferable. It is likely that for all the naturally occurring antibacterials there is some or other mechanism of resistance present in nature. Excess use of an antibiotic creates selection pressure to enrich resistant population who acquired resistance genes. Albomycin or other sideromycin would enrich the population lacking functional ferrichrome transport system, what is a non-transferable trait. This will reduce the common danger of spreading resistance, as is common for other antibiotic resistance. The development of one step complete resistance by spontaneous transport negative mutation decreases the pressure to develop other types of resistance mechanisms. Under selection bacteria continuously evolve resistance to antimicrobial drugs, including multidrug resistance. It is unavoidable as this brings fittness and is an aspect of general evolution of bacteria that is unstoppable. Therefore, the only means of dealing with this situation is to either delay the emergence of resistance or restrict subsequent dissemination of resistant bacteria or resistance genes. Resistance to antimicrobial drugs in bacteria can result from mutations in target housekeeping genes, which could be slow depending on the selection pressure and fitness of resistant gene product, but inevitable so far. Alternatively the resistance feat can be bought in by horizontal acquisition of foreign resistance genes providing means of detoxifying the antibacterial. Almost invariably all target site resistant mutants develop slowly and in steps, posing from low to intermediate to high level of resistance with the progress of time. Each step of resistance provide a stage for further development of the resistance trait and their dissemination. Such step wise resistance development can clearly be avoided with sideromycin type antibacterials which gives only yes-no option to resistance. If a cell is able to bring in the sideromycin, it will keep accumulating against an apparent concentration gradient (active transport). In transport negetive mutants cellular target would not at all face a selection pressure to evolve resistance. Thus development of target site resistance could be minimized.

4.5 Resistance cost competitiveness

Disposing ferrichrome transport leads to albomycin resistance, but at the cost of a compromised iron supply. Under isolated in vitro culture conditions it is difficult to determine the contribution of a specific transport system on overall fitness of the species, it can be determined in an in vivo mixed infection model. In the murine infection model resistant mutants were found to be less competitive than the wild type. Thus disposing off ferrichrome transport can give advantage under albomycin selection pressure temporarily, but cripples the virulence giving the immune system better chance to clear the infection. Thus a mixed infection will always thwart mutants without selection pressure, thereby limiting spread of resistance.

4.6 An effective strategy to win the battle against bad bugs

Sideromycins can accommodate structurally very different antibiotic moieties conjugated to any iron chelating siderophores. As in albomycin the antibiotic moiety is a sulphur containing nucleoside, whereas antibacterial activity of salmycin is posed by a disaccharide moiety. The ferric hydroxamate transport system in *S. pneumoniae* offers means to develop antibiotics with ferric hydroxamates as carriers. Albomycin and salmycin are promising examples of how the structures of such chemically synthesized antibiotics might appear. The ferric hydroxamate transport system tolerates chemically different hydroxamates with different ligands. The need for iron acquisition made transport negative mutants less virulent in mixed infection. In addition, the surface-exposed iron transport lipoproteins are strong antigens and their use for active and passive immunization protects mice against invasive *S. pneumoniae* disease (Brown *et al.*, 2001; Jomaa *et al.*, 2005; Whalan *et al.*, 2005). During microbial infection of a mammalian host, iron availability is lowered and the iron uptake systems of pathogens are induced. The idea of conjugating an antibiotic with a siderophore to selectively target a pathogen by carrying the drug into pathogens through microbial iron uptake systems is exploitable for designing drug delivery strategies in future. This work provided evidence that siderophore-mediated drug delivery (the Trojan Horse approach) can practically be used against bacterial infection. The ideal siderophore and drug combination could be found by a trial and error method. Till then, study of natural sideromycins can provide directions how a multitude of species-directed or broadly active conjugates may look and work like in the future.

5. Summary

Albomycin overproduction was standardized and mid-scale fermentation was carried out. From the fermentation broth albomycin was recovered to homogeneity by three-step chromatography. This purified albomycin was used throughout this study. The activity spectrum of albomycin against common bacterial pathogens was determined. Correlation of ferrichrome utilization and albomycin sensitivity was verified. Intrinsic albomycin resistance of *Proteus* and *Morganella* attributed to the lack of a ferrichrome transport system, unlike other enterobacteriaceae tested. Pseudomonads and *Haemophilus* related generas were also intrinsically resistant. Among gram positives, coagulase positive staphylococci, *Bacillus subtilis* and pneumococci were highly sensitive to albomycin.

Reasons of pneumococcal sensitivity to albomycin and salmycin were determined. Competitive cross-feeding of sideromycins with hydroxamate siderophores and proteolytic cleavage experiments provided evidence that albomycin is transported in pneumococci through a hydroxamate transport system. Targeted mutagenesis (Insertion Duplication and Deletion) were done to identify the involvement of the putative iron transport systems in the R6 strain. This approach identified the gene for the FhuD binding protein of the transport system. Complementation by ectopic insertion confirmed the role of *fhuD* in hydroxamate transport in pneumococcus. To identify the other components of the hydroxamate transport machinery, an in vitro random mutagenesis method was standardized. From a randomly mutagenized library transport negative mutants were picked. From these mutants the *fhuB* and the *fhuG* genes were identified.

Recombinant FhuD was cloned and overexpressed. This FhuD is a lipoprotein that is able to bind a wide variety of hydroxamate substrates but not free $Fe^{2+/3+}$ or haemin. Substrate binding leads to conformational changes conferring partial resistance to proteolytic attack. A 3D homology model of Sp-FhuD was made based on *E. coli* FhuD crystal structure. 3D alignment identified both similarity and minor variations at the substrate binding pocket.

Other than in vitro, efficacy of albomycin was tested in a murine infection model. After 6 h of single dose antibiotic administration, albomycin performed better than equivalent dose of gentamycin to reduce bacterial load in *Y. enterocolitica* infected mice spleen. Albomycin was highly effective against murine pneumococcal infections. All albomycin treated mice recovered from pneumococcal infection. In a mixed infection model albomycin resistant mutants showed a compromised virulence.

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7. Abbreviations

µ	micro
^{0}C	degree Centigrade
3D	Three Dimension
ABC	ATP binding cassette
Alb	Albomycin
Amp	Ampicillin
Amx	Amoxicillin
APS	Ammonium Per Sulfate
ATCC	American Type Culture Collection
ATP	Adenosine-Tri-Phosphate
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide-nucleotide BLAST
BLASTP	Protein-protein BLAST
BLASTX	Protein- oligonucleotide 6 frame translated protein BLAST
bp	base-pair
Bs, *B. subtilis*	*Bacillus subtilis*
BSA	Bovine Serum Albumin (fraction V)
C+Y	C-medium for pneumococcus with Yeast extract
CFU	Colony Forming Unit
CI	Competitive Index
CLUSTALW	Command line multiple sequence alignment by software tool
CM	Cytoplasmic Membrane
d	day
Da	Dalton
DNA	Deoxy-ribo Nucleic Acid
dNTP	deoxy-Nucleotide Tri-Phosphate
Ec, *E. coli*	*Escherichia coli*
EDDHA	Ethylene-Diamine-Di-(o-Hydroxyphenylacetic Acid)
EDTA	Ethylene-Diamine-Tetra-Acetic acid
Erm	Erythromycin
Fer	Ferrichrome
fhu	Ferric Hydroxamate Uptake

Fox	Ferrioxamine
fur	Ferric Uptake Regulator
FURTA	Fur-Titration Assay
g	gram
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
h	hour
H. influenzae	*Haemophilus influenzae*
HA	*Streptomyces* sporulation medium
HPLC	High Pressure Liquid Chromatography
IDM	Insertion Duplication Mutagenesis
IPTG	Iso-propyl-thio-galactoside
k	kilo
kb	Kilo-Base pair
kD	Kilo-Dalton
l	litre
m	milli
M	Molar
ME	Mosaic End sequence
MIC	Minimum Inhibitory Concentration
min	minute
mm	milli-meter
MOPS	Morpholino-Propane Sulphonic acid
n	nano
NB	Nutrient Broth
NBD	Nutrient Broth with Dipyridyl
NCBI	National Center for Biotechnology Information
NTA	Nitrilo-Tri-Acetate
OD	Optical Density
OM	Outer Membrane
ORF	Open Reading Frame
P. vulgaris	*Proteus vulgaris*
PAGE	Poly Acrylamide Gel Electrophoresis
PBP	Periplasmic Binding Protein

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
Pm, *P. Mirabilis*	*Proteus mirabilis*
PMSF	Phenyl-Methyl-Sulfonyl-Fluoride
RBS	Ribosomal Binding Site
RNA	Ribo Nucleic Acid
rpm	revolutions per minute
S. agalactiae	*Streptococcus agalactiae*
S. pyogenes	*Streptococcus pyogenes*
Sa, *S. aureus*	*Staphylococcus aureus*
Sal	Salmycin
SDS	Sodium Dodecyl Sulphate
sec	second (time)
Sn	Streptonigrin
Sp, *S. pneumoniae*	*Streptococcus pneumoniae*
sp.	species
TAE	Tris-Acetate-EDTA
TCA cycle	Tri-Carboxylic-Acid cycle
TCA	Tri-Chloro-acetate
TE	Tris-EDTA
TEMED	N,N,N'-tetramethylethylendiamine
Tet	Tetracycline
THY	Todd-Hewitt broth with Yeast Extract
Tn5	Transposon 5
t-RNA	transfer-RNA
TY	Tryptone-Yeast extract medium
UV	Ultra-Violet
V	Volt
x g	gyration
Y. enterocolitica	*Yersinia enterocolitica*

Publications :

1. Avijit Pramanik, Volkmar Braun. Albomycin uptake via a ferric hydroxamate transport system of *Streptococcus pneumoniae* R6.
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2. Avijit Pramanik, Uwe H. Stroeher, Juliane Krejci, Alistair Standish, Erwin Bohn, James C. Paton, Ingo B. Autenrieth and Volkmar Braun. Albomycin as an effective antibiotic against *Yersinia enterocolitica* and *Streptococcus pneumoniae*. Manuscript submitted.

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