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EVOLUTION OF BETA- LACTAM RESISTANCE IN *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

K. pneumoniae is recognized as a common opportunistic pathogen. Numerous reports have been published worldwide on outbreaks in different healthcare settings. *K. pneumoniae* is inherently resistant to penicillins, including semi-synthetic broad-spectrum penicillins. The drug of choice for empirical treatment is often a cephalosporin. However, the use of cephalosporins is known to select for extended-spectrum beta-lactamase (ESBL)-producing strains.

The focus of this thesis is the beta-lactamase gene in *K. pneumoniae*, and its relationship to beta-lactamase genes present in plasmids in gram-negative bacteria. In Paper I, the intention was to identify presumed beta-lactamase SHV-1-encoding plasmids in fecal *Klebsiella* isolates from neonates in Swedish special care units. No such plasmids were detected, however. Instead, a chromosomal beta-lactamase gene was identified in all *K. pneumoniae*, but in none of the *Klebsiella oxytoca* isolates. This species-specific gene was seen in 10 allelic variants; some closely related to the prototypic plasmid-borne SHV-1 gene, indicating that an allelic variant of the *K. pneumoniae* chromosomal beta-lactamase gene is the ancestor of the plasmid-borne SHV-encoding genes. In Paper II, the observed diversity of the chromosomal *K. pneumoniae* beta-lactamase gene was further investigated in order to study its evolution in relation to the three phylogenetic groups of *K. pneumoniae*. Three sequence groups, corresponding to the phylogenetic groups, were identified, bla_{SHV} , bla_{LEN} , and bla_{OKP} . In Paper III, the genetic context of bla_{SHV} in *K. pneumoniae* chromosomes and plasmids from various gram-negative bacteria was analyzed. Plasmid-borne bla_{SHV} genes were found to be surrounded by DNA highly similar to the *K. pneumoniae* chromosome. IS26 elements flanked the bla_{SHV} regions. Nine distinct junctions between IS26 and *K. pneumoniae* chromosomal DNA, and seven different region-lengths were identified. In contrast to a high diversity observed among chromosomal sequences, only two groups of plasmid sequences were seen.

This thesis has demonstrated that only one of three ancient *K. pneumoniae* chromosomal beta-lactamase gene families, bla_{SHV} , is found on plasmids. This is possibly the result from a single IS26 mediated mobilization of bla_{SHV} and surrounding DNA from *K. pneumoniae*. The two groups of plasmid bla_{SHV} regions seen today could be the result of post-mobilization evolution involving size reductions and nucleotide substitutions. We conclude that mobilization of bla_{SHV} from *K. pneumoniae* chromosomes is not a driving force in the emergence of resistance in response to beta-lactam therapy. The spread is more likely a consequence of mobilization of IS26 flanked bla_{SHV} regions between plasmids, and mobilization of plasmids between different bacteria.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. **Hæggman, S.**, Löfdahl, S., and Burman, L.G.
An allelic variant of the chromosomal gene for class A β -lactamase K2, specific for *Klebsiella pneumoniae*, is the ancestor of SHV-1
Antimicrobial Agents and Chemotherapy, 1997, 41(12): 2705-2709
- II. **Hæggman, S.**, Löfdahl, S., Paauw, A., Verhoef, J., and Brisse, S.
Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*
Antimicrobial Agents and Chemotherapy, 2004, 48(7): 2400-2408
- III. **Hæggman, S.**, and Löfdahl, S.
Low initial transposition frequency of chromosomal bla_{SHV} and subsequent evolution have formed the present population of acquired bla_{SHV}
Manuscript

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LIST OF ABBREVIATIONS

bp	Base pairs
ESBL	Extended-spectrum beta-lactamase
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction

1 INTRODUCTION

Antibiotic resistance is a serious problem in clinical medicine. For example, the efficacy of treatment with the widely used beta-lactam antibiotics is constantly challenged by the emergence of new resistant bacterial strains.

Penicillin, the first industrially produced beta-lactam antibiotic, has been in clinical use since the 1940s. Soon after its introduction into clinical praxis, however, resistant bacterial strains emerged. New chemically modified beta-lactam antibiotics were therefore successively developed by the pharmaceutical companies. The broad-spectrum penicillins were soon followed by a large number of cephalosporins. Cefotaxime, the first so called “third generation” cephalosporin was introduced in the early 1980s and it is still one of the most widely used cephalosporins.

In gram-negative bacteria, beta-lactamase production is considered the main antibiotic resistance mechanism. Beta-lactamases are enzymes that inactivate beta-lactam antibiotics. This group of enzymes comprises many variants with different spectra of activity. New variants are continually being identified around the world, the most worrisome being the extended-spectrum beta-lactamases (ESBLs) belonging to the enzyme families TEM, SHV, and CTX-M.

Genes encoding beta-lactamases are found both in bacterial chromosomes and plasmids. Since the 1980s there have been numerous reports from different healthcare settings worldwide on outbreaks caused by ESBL-producing pathogenic gram-negative bacteria. Many of these reports involve *Klebsiella pneumoniae*, which is recognized as one of the most common causes of healthcare associated bacterial infections.

The subject of this thesis is beta-lactam resistance in *K. pneumoniae*, with focus on the inherent chromosomal beta-lactamase gene. I have investigated its diversity, evolution, and genetic context. I have also compared it to plasmid-borne SHV genes in order to study evolutionary relationships between chromosomal and plasmid-borne SHV beta-lactamase genes, and to clarify mechanisms involved in the worldwide dissemination of different SHV ESBL-encoding genes.

2 KLEBSIELLA PNEUMONIAE

2.1 PHYSIOLOGY

Klebsiella pneumoniae is a non-motile gram-negative rod-shaped bacterium, $\leq 6 \mu\text{m}$ long and $\leq 1 \mu\text{m}$ in diameter, which can grow both aerobically and anaerobically (Brisse 2006). The cells are often surrounded by a polysaccharide capsule, which prevents phagocytosis. The capsule is regarded as a virulence determinant, and most clinical isolates are capsulated (Favre 1999). There are 77 capsular (K) serotypes described, and some of them have been associated with severe infections in humans and animals (Brisse, Fevre et al. 2009). *K. pneumoniae* is a common member of the human intestinal flora, and it is said to be ubiquitous, meaning that it can be found almost everywhere, for example also in water, soil, and plants (Podschun, Pietsch et al. 2001), (Brisse and Duijkeren 2005). Some strains isolated from plants are nitrogen-fixing and of interest since they can increase plant growth under agricultural conditions. One of the three publically available *K. pneumoniae* whole genome sequences is that of a nitrogen-fixing strain isolated from the interior of nitrogen-efficient maize plants (Fouts, Tyler et al. 2008).

2.2 TAXONOMY

K. pneumoniae belongs to the family *Enterobacteriaceae* (Francino 2006); (Grimont PAD 2005; Brisse 2006). It is the type species of the genus *Klebsiella*, which was named in honor of the German microbiologist Edwin Klebs who lived 1834-1913 (Trevisan 1885). The first *Klebsiella* strain ever described was a capsulated bacillus isolated from a patient with rhinoscleroma (Frisch 1882).

The type strain of *K. pneumoniae* is ATCC 13883 (NCTC 9633, CDC 298-53, CIP 82.91). This strain belongs to one of three subspecies, namely *pneumoniae*. The other two *K. pneumoniae* subspecies are *ozaenae* and *rhinoscleromatis*. The definitions of the subspecies are not based on genomic distinctness but on pathogenesis criteria (Brisse 2006).

The nomenclature for organisms within the genus *Klebsiella* has been confusing. For example, the existence of an indole positive species, now known as *Klebsiella oxytoca*, was questioned. It was then regarded as a

biogroup of *K. pneumoniae* (Edwards and Ewing 1972), (Ørskov 1974). By DNA relatedness studies, however, it was clarified that *K. oxytoca* is distinct from *K. pneumoniae* at the species level (Jain 1974), (Brenner 1977).

When the genetic diversity of the species *K. pneumoniae* was investigated, three sequence clusters, or phylogenetic groups, were identified (Brisse and Verhoef 2001). The groups were named KpI, KpII, and KpIII. Recently, it has been shown that even more phylogenetic groups exist within *K. pneumoniae* (Jonas, Spitzmuller et al. 2004).

Most *K. pneumoniae* infections are caused by strains belonging to the phylogenetic group KpI (Brisse 2004).

2.3 HEALTHCARE-ASSOCIATED INFECTIONS

K. pneumoniae is recognized as a common opportunistic pathogen. It accounts for a significant proportion of healthcare-associated, or nosocomial, infections that are frequently caused by gram-negative enterobacteria (Podschun and Ullman 1998a). In many studies, it is one of the three most common gram-negative pathogens, together with *E. coli* and *Pseudomonas aeruginosa* (Richards 2000), (Garcia de la Torre, Romero-Vivas et al. 1985), (Williams and Thomas 1990). Age is one of the predisposing factors, i.e., very young or very old (Feldman 1990). The reservoir for the *K. pneumoniae* strains is often the intestinal tracts of the patients.

Numerous reports have been published worldwide on outbreaks caused by *K. pneumoniae* in different healthcare settings, like neonatal wards, nursing homes, and intensive care units (Liu, Gur et al. 1992), (Arpin, Dubois et al. 2003), (Gniadkowski, Palucha et al. 1998), (Livermore and Yuan 1996), (Babini and Livermore 2000), (Sadowski and al. 1979), (Lytsy, Sandegren et al. 2008).

2.4 ANTIBIOTIC TREATMENT

K. pneumoniae is inherently resistant to penicillins, including semi-synthetic broad-spectrum penicillins. Therefore, the drug of choice for empirical treatment is often a cephalosporin. However, the use of cephalosporins is known to select for resistant *K. pneumoniae* strains

(Livermore 1991), (Bedenic 2002). This is of great concern in human healthcare around the world. The number of *K. pneumoniae* strains producing ESBL variants of the widespread plasmid-encoded beta-lactamases belonging to the enzyme families TEM, SHV, and CTX-M are constantly increasing (Bradford 2001), (Jacoby and Munoz-Price 2005), (Paterson, Hujer et al. 2003), (Paterson and McCormack 2003), (Steward, Rasheed et al. 2001), (Winokur, Canton et al. 2001).

3 BETA-LACTAM ANTIBIOTICS

All beta-lactam antibiotics contain a beta-lactam ring, hence the name (Rolinson and Geddes 2007). This molecular structure is the target for beta-lactamases, which by hydrolysis open the ring and thereby inactivate the antibiotic (Fig. 1).

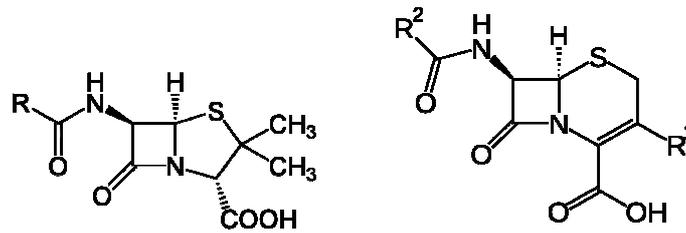


Fig. 1. The core structure of penicillins (left) and cephalosporins (right). The four-membered ring is the beta-lactam ring. The five-membered ring in penicillins is a thiazolidine ring. The six-membered ring in cephalosporins is a dihydrothiazine ring. The R indicates various side groups in different penicillins, e.g., a benzyl group in penicillin G. R¹ and R² indicate positions for various side groups in different cephalosporins.

The four major groups of beta-lactam antibiotics are penicillins, cephalosporins, carbapenems, and monobactams (Table 1). The beta-lactamase gene in focus of this thesis exists in variants which encode enzymes that mainly inactivate penicillins and cephalosporins.

Table 1. Examples of beta-lactam antibiotics in the four major groups

Group	Beta-lactam antibiotic(s)	Commonly referred to as
Penicillins	Penicillin G, penicillin V	
	Ampicillin, piperacillin	Broad-spectrum penicillins
Cephalosporins	Cephalothin	First generation cephalosporins
	Cefuroxime	Second generation cephalosporins
	Cefotaxime, ceftazidime	Third generation cephalosporins
Carbapenems	Imipenem, meropenem	
Monobactams	Aztreonam	

3.1 HISTORY

Beta-lactam antibiotics have been used as therapeutic agents since the 1940s. They are excellent drugs because they are non-toxic and well tolerated by most patients.

Less than one hundred years ago, in the 1920s, Sir Alexander Fleming made the ground-breaking discovery that a certain *Penicillium* mould produced a powerful antibacterial substance (Flemming 1929). He named the filterable active agent penicillin, and reported that the action of penicillin was marked on the pyogenic cocci and the diphtheria group of bacilli, and that for example the coli-typhoid bacteria were quite insensitive. Fleming, however, was not able to purify penicillin. It was not until the late 1930s that this was accomplished (Chain 1938).

Penicillin G (benzylpenicillin) was the first beta-lactam antibiotic in clinical use (Florey and Florey 1943). A major drawback of this drug is that it cannot be administered orally due to its lack of stability in the acid stomach. In the 1950s, penicillin V (phenoxymethylpenicillin), was developed (Brandl, Giovannini et al. 1953). This semi-synthetic derivative is acid stable. Both penicillin G and V, however, have rather limited spectrum of activity, and are not suitable for treating infections caused by gram-negative bacteria.

Ampicillin is a broad-spectrum penicillin that was developed in the 1960s by chemical modification of the side chain of the beta-lactam ring of benzylpenicillin (Rolinson and Stevens 1961). Its spectrum of activity includes gram-negatives like *E. coli*. Ampicillin has been followed by many other beta-lactam antibiotics, which have been developed in order to further increase the spectrum of activity.

A wide range of semi-synthetic beta-lactam antibiotics have been developed by pharmaceutical companies in response to the emergence of resistant bacterial strains, which were soon selected for by the therapeutic use of every new beta-lactam antibiotic (Livermore 2009).

Cephalosporins are beta-lactam antibiotics originally isolated from the mould *Cephalosporium* (Murray, Rosenthal et al. 2009). They differ from penicillins in having a dihydrothiazine ring fused with the beta-lactam ring (Fig. 1). This gives more opportunities for biochemical modifications (at positions R¹ and R²), in order to expand the spectrum

of activity and improve the pharmacokinetic properties of the drug. Cephalosporins, in general, have enhanced activity against gram-negative bacteria. Commonly used semi-synthetic cephalosporins are cefuroxime, cefotaxime, and ceftazidime (Knothe and Dette 1983).

3.2 MECHANISMS OF ACTION

Beta-lactam antibiotics only kill growing bacteria. They bind so called penicillin binding proteins (PBPs), which are enzymes involved in cell wall synthesis (Sauvage, Kerff et al. 2008). The PBPs are located on the outer side of the cytoplasmic membrane. In gram-negative bacteria this is in the periplasm. Some PBPs are transpeptidases that catalyze the cross-linking of glycan strands in the nascent peptidoglycan. When these enzymes are inactivated cell wall synthesis becomes severely disturbed, which leads to cell lysis.

The mechanism of action is explained by the structural similarity between the beta-lactam ring and the peptidoglycan building block acyl-D-alanyl-D-alanine (Tipper and Strominger 1965). A covalent bond formed between the beta-lactam ring and an active site serine residue in the PBP results in inactivation of the PBP.

It is the fact that peptidoglycan, the major structural component of most bacterial cell walls, is unique and essential for bacteria that makes beta-lactam antibiotics excellent non-toxic drugs for humans.

4 BETA-LACTAMASES

Resistance to beta-lactam antibiotics can have different causes (Table 2).

Table 2. The three major beta-lactam resistance mechanisms

Mechanism	Effect on the antibiotic	Reference
Loss of outer membrane proteins	Prevented from reaching its site of action	(Nikaido 2003)
Altered PBPs	Prevented from binding target enzyme	(Spratt 1994)
Beta-lactamase production	Inactivated irreversibly	(Jacoby and Munoz-Price 2005)

Production of one or more beta-lactamases is by far the most common mechanism for beta-lactam resistance among gram-negative bacteria (Livermore 2009). *K. pneumoniae* strains, for example, often carry plasmids producing one or more beta-lactamase variants (Pitout and Laupland 2008).

4.1 ENZYME CHARACTERISTICS

Beta-lactamases hydrolyze the amide bond of the beta-lactam ring (Ghuysen 1991; Livermore 2009). The molecular mass of these enzymes is ~30 kDa (~280 amino acid residues). In gram-negative bacteria, beta-lactamases are found as soluble proteins in the periplasm. Beta-lactamases and PBPs are structurally related and share certain mechanistic features (Massova and Mobashery 1998). These enzymes probably have a common origin (Massova and Mobashery 1999; Gniadkowski 2008).

Some beta-lactamases are zinc-dependent enzymes. Metal-independent beta-lactamases are, however, more common. They contain an active site serine residue to which the antibiotic is covalently bound, via an ester bond, as a catalytic intermediate. The ester bond is subsequently hydrolyzed and the inactivated antibiotic is released from the enzyme. The beta-lactamase is then ready for a new catalytic cycle. This mechanism is analogous to the binding of beta-lactam antibiotics to PBPs. The main difference is that the covalent bond formed between

the antibiotic and the active site serine in the PBP is not, or very slowly, hydrolyzed. Therefore, the enzyme activity of the PBP is blocked.

Beta-lactamase activity can be demonstrated by using, for example, nitrocefin, a chromogenic cephalosporin reagent (Gallenì and Frère 2007).

Beta-lactamase activity can be inhibited by clavulanic acid, a beta-lactam compound that was discovered in 1977 (Reading and Cole 1977). This compound and other beta-lactamase inhibitors (sulbactam and tazobactam) are used in combination with beta-lactam antibiotics as therapeutic agents. For example, the drug Augmentin contains a mixture of amoxicillin and clavulanic acid.

4.2 CLASSIFICATION

A large number of beta-lactamase variants have been identified since the report in 1940 on an *E. coli* enzyme able to destroy penicillin (Abraham and Chain 1940). Different classification schemes for these enzymes have been presented. The most recent scheme was published this year (Bush and Jacoby 2010). The first attempts to classify beta-lactamases were based on functional and biochemical characteristics of the enzyme, like, substrate profile and isoelectric point. Later, amino acid sequence information was added to the schemes (Bush, Jacoby et al. 1995).

The latest classification schemes for beta-lactamases include four molecular classes, A, B, C, and D, based on amino acid sequence information. They also include four functional groups, 1 to 4, which are based on hydrolytic and inhibition properties of the enzymes. The inhibitors used in the latest classification scheme are clavulanic acid, tazobactam and EDTA.

Molecular class A, C, and D comprise the serine beta-lactamases, and class B the zinc-dependent metalloenzymes. Class A and D include penicillinases and cephalosporinases, most of which are inhibited by clavulanic acid or tazobactam. Class C include cephalosporinases that are not, or poorly, inhibited by clavulanic acid or tazobactam. Class B comprises the metalloenzymes, which are inhibited by EDTA and have carbapenems as distinctive substrates. Representative enzymes of the different molecular classes are presented in Table 3.

Table 3. Classification of common beta-lactamases

Molecular class	Functional group	Beta-lactamase(s)
C	1	AmpC (<i>E. coli</i>)
A	2b	TEM-1, TEM-2, SHV-1
	2be	TEM-3, SHV-2, CTX-M-15
	2br	TEM-30, SHV-10
	2ber	TEM-50
D	2d	OXA-1
	2de	OXA-11
	2df	OXA-23
	2f	KPC-2
B	3a	IMP-1, VIM-1

4.3 NOMENCLATURE

The nomenclature of beta-lactamases is comprehensively covered in a recent minireview (Jacoby 2006). The enzymes have been named after, for example, biochemical properties, the strain producing it, the person first characterizing it, or the patient providing the first sample. Table 4 presents examples of beta-lactamases found in *K. pneumoniae*.

Table 4. Some beta-lactamases found in *K. pneumoniae*

Beta-lactamase	Derivation of name	Reference
SHV	Sulfhydryl reagent variable	(Matthew, Hedges et al. 1979)
LEN	From <i>K. pneumoniae</i> strain LEN-1	(Arakawa, Ohta et al. 1986)
OKP	Other <i>K. pneumoniae</i> beta-lactamase	(Paper II)
TEM	Named after the patient (Temoneira) providing the first sample	(Datta and Kontomichalou 1965)
CTX-M	Active on cefotaxime, first isolated in Munich	(Bauernfeind, Grimm et al. 1990)
KPC	<i>K. pneumoniae</i> carbapenemase	(Yigit, Queenan et al. 2001)

Some of the enzymes show many closely related variants, which form enzyme families. Such families are TEM, SHV, and CTX-M. At present there are for example more than 130 SHV variants described. See publically available databases for updated information (<http://www.lahey.org/Studies/>; http://www.pasteur.fr/recherche/genopole/PF8/betalact_en.html).

For clarity, when mentioning an ESBL, it is suggested to always include the enzyme family name, for example SHV ESBL (Livermore 2008).

4.4 BETA-LACTAMASES IN *K. PNEUMONIAE*

K. pneumoniae is inherently resistant to penicillins and early cephalosporins due to constitutive production of a chromosomally encoded class A group 2b beta-lactamase (Petit, Ben-Yaghlane-Bousslama et al. 1992), (Paper I). In addition to this enzyme, many *K. pneumoniae* strains produce one or more plasmid-mediated beta-lactamases. The most common belong to the enzyme families TEM, SHV, and CTX-M (Jacoby 1997), (Gniadkowski 2008), (Hawkey 2008), (Elhani, Bakir et al. 2010). SHV ESBLs have been demonstrated in *K. pneumoniae* since 1983 (Knothe, Shah et al. 1983), (Kliebe, Nies et al. 1985). Today, the most common SHV ESBLs worldwide are SHV-2, SHV-5 and SHV-12 (Hrabak, Empel et al. 2009). New SHV variants still emerge (Jones, Tuckman et al. 2009). In recent years, reports on plasmid-mediated enzymes belonging to the CTX-M family have become more and more frequent (Livermore, Canton et al. 2007).

5 IDENTIFICATION OF *K. PNEUMONIAE* CHROMOSOMAL BETA-LACTAMASE GENES

5.1 PAPER I

An allelic variant of the chromosomal gene for class A beta-lactamase K2, specific for *Klebsiella pneumoniae*, is the ancestor of SHV-1

The *Klebsiella* strains used in this study were 172 fecal non-duplicate isolates from neonates in 22 Swedish special care units. These strains had been included in two previous studies; one focusing on the epidemiology of strains of different *Enterobacteriaceae* species (Tullus, Berglund et al. 1988), and one focusing on the epidemiology of the plasmid-mediated beta-lactamases TEM-1, OXA-1, and SHV-1 (Burman, Haeggman et al. 1992). Also 15 international reference strains, including the type strains of *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, *Klebsiella oxytoca*, *K. planticola*, and *K. terrigena* were included in the study.

The aim of the study was to identify presumed SHV-1-encoding plasmids among the fecal *Klebsiella* isolates and to find out whether SHV-1-encoding genes were carried by promiscuous plasmids or not. However, in Southern blot analyses of plasmid DNA preparations, we failed to detect any hybridization between plasmid bands and the SHV-1 probe used. This probe, a gel purified 352 bp *PvuII* intragenic fragment obtained by digestion of plasmid pMON38, was the one used in the previous colony hybridization assay (Burman, Haeggman et al. 1992). The recombinant plasmid pMON38 is derived from the SHV-1-encoding plasmid R974 (Mercier and Levesque 1990).

To increase the sensitivity of our Southern blot hybridizations, we tested another type of probe – a PCR amplicon. The PCR primers designed for this purpose were based on the sequence of the intragenic 352 bp fragment of SHV-1_{R974} present in pMON38, which was used as PCR template. This PCR yielded a 231 bp amplicon which was labeled with [α -³²P]dCTP and used as probe in subsequent Southern blot analyses. Also this probe failed to hybridize with plasmid DNA. By using the PCR-derived probe the hybridization signal was increased and the background was decreased. However, weak hybridization signal was seen with chromosomal DNA present in low concentration in some of

the plasmid preparations. This finding prompted us to perform Southern blot analysis of genomic DNA. By doing this, we detected hybridization with chromosomal DNA from all tested *K. pneumoniae* strains – 20 fecal isolates and three reference strains (ATCC 13883T, 1976E, and LEN-1).

The developed SHV-1-based PCR was used to screen 187 *Klebsiella* strains. This revealed the presence of an SHV-1 or SHV-1-related beta-lactamase gene in all 116 *K. pneumoniae* strains included in the study, and the lack of such a gene in all 69 *Klebsiella oxytoca* strains as well as in the *K. planticola* and *K. terrigena* type strains.

The finding in *K. pneumoniae* of what appeared to be a species-specific beta-lactamase gene, closely related to the plasmid-borne SHV-1 beta-lactamase gene, was rather unexpected. At that time SHV-1 was generally regarded as a plasmid-mediated beta-lactamase (Bush, Jacoby et al. 1995), (Fuster, Roy et al. 1993) even though some reports had indicated that occasional *K. pneumoniae* strains could produce chromosomally encoded SHV-1-like enzymes (Matthew and Harris 1976), (Nugent and Hedges 1979), (Petit, Ben-Yaghlane-Bouslama et al. 1992). The only *K. pneumoniae* chromosomal beta-lactamase gene that had been identified and sequenced at that time was the one encoding beta-lactamase LEN-1 (Arakawa, Ohta et al. 1986). Both SHV and LEN enzymes are distinct from AmpC, i.e., the chromosomal beta-lactamase produced by *E. coli* and many other species of *Enterobacteriaceae*.

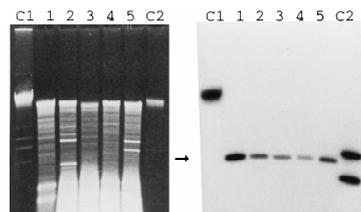


Fig. 2. Restriction fragment length polymorphism analysis of DNA from five fecal *K. pneumoniae* isolates. Agarose gel of total bacterial DNA preparations digested with *Bgl*II (left), and the corresponding autoradiogram after Southern blot hybridization (right). Undigested DNA preparations of *K. pneumoniae* 1976E (C1) and *E. coli* J53-2 containing pMON38 (C2). The position of the *bla*_{SHV}-positive ~5-kb *Bgl*II fragment is indicated by an arrow. (Adapted from Paper I).

Further characterization of *K. pneumoniae* genomic DNA, using restriction fragment length polymorphism analysis, showed that the beta-lactamase gene was located within a conserved chromosomal region. The SHV-1 PCR probe hybridized to a ~5 kb *Bgl*II fragment in all tested strains (Fig. 2).

The negative results of conjugation experiments performed were also in support of the notion of a chromosomal location of the SHV-1-like beta-lactamase gene in *K. pneumoniae*. Transfer of ampicillin resistance was only demonstrated for one of the tested isolates. This was the only *K. pneumoniae* isolate that had been colony hybridization positive for both SHV-1 and TEM-1.

Analytical isoelectric focusing of chromosomally encoded *K. pneumoniae* beta-lactamases identified two major groups. Enzymes with isoelectric point (pI) 7.6 were the most common. They were produced by isolates collected from neonates in 18 of the 22 special care units. Enzymes focusing at pI 7.1 were produced by isolates from 8 special care units only. A pI of 7.6 is characteristic for SHV-1 beta-lactamase and pI 7.1 is characteristic for LEN-1. Other pIs were also detected (for details see Table 1, Paper I).

Randomly selected SHV-1-PCR amplicons were subjected to DNA sequencing. Alignment of sequences and tree analysis demonstrated the same groupings as seen by isoelectric focusing. Sequences from strains producing a pI 7.6 enzyme formed one cluster, as did the strains producing pI 7.1 enzymes (Fig. 4, Paper I).

Minimal inhibitory concentrations (MICs) were determined for ampicillin, ampicillin in the presence of clavulanic acid, piperacillin, cephalothin, cefotaxime, and aztreonam. All strains exhibited broad-spectrum beta-lactamase activity, and the beta-lactamase inhibitor clavulanic acid markedly lowered the ampicillin MICs. There were no distinctions between the *K. pneumoniae* isolates producing SHV-1-like beta-lactamase and the ones producing LEN-1-like enzymes. No extended-spectrum beta-lactamase activity was detected.

In summary, what started as a search for plasmids carrying SHV-1 beta-lactamase genes resulted in the identification of a chromosomal beta-lactamase gene present in all *K. pneumoniae*, i.e., a species-specific beta-lactamase gene. In our material, this gene was seen in 10 allelic variants.

Most of the variants were closely related to the prototypic plasmid-borne SHV-1 beta-lactamase gene. This made us propose that an allelic variant of the *K. pneumoniae* chromosomal beta-lactamase gene is the ancestor of the plasmid-borne SHV-encoding genes observed frequently in *K. pneumoniae* and other pathogenic gram-negative bacteria.

6 DIVERSITY OF *K. PNEUMONIAE* CHROMOSOMAL BETA-LACTAMASE GENES

6.1 PAPER II

Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*

Paper II reports extended analysis of the evolutionary relationships between different chromosomal beta-lactamase gene variants. The aim of this study was to investigate whether beta-lactamase diversification has occurred as part of a natural, long-term evolutionary process, or whether the presence of a chromosomal beta-lactamase gene in *K. pneumoniae* is the result of a recent horizontal acquisition followed by clonal expansion in response to selective pressure of various beta-lactam antibiotics used in clinical medicine.

Twenty *K. pneumoniae* strains were randomly selected from the two major groups identified in our previous study (Paper I); ten of them produced a pI 7.6 SHV beta-lactamase and the other ten produced a pI 7.1 LEN beta-lactamase. One of the two strains found to have a beta-lactamase gene different from *bla*_{SHV} and *bla*_{LEN} was also included. The excluded strain was negative in Southern blot analysis. In addition to these 21 strains, we included seven strains from a previous taxonomic study of *K. pneumoniae*, in which three *K. pneumoniae* phylogenetic groups, KpI, KpII, and KpIII, were identified (Brisse et al. 2001).

We studied the nucleotide diversity and evolution of the *K. pneumoniae* chromosomal beta-lactamase gene and two housekeeping genes; *gyrA*, coding for subunit A of gyrase, and *mdh*, coding for malate dehydrogenase. The *gyrA* gene had previously been used as a marker for the three *K. pneumoniae* phylogenetic groups (Brisse et al. 2001).

A 789-bp portion of the beta-lactamase genes was sequenced from the 28 strains included in this study, i.e., a larger part than in our previous study (Paper I) in which we sequenced 231-bp PCR amplicon. The obtained sequences formed three distinct groups. This corresponded fully to our earlier results (Paper I). One of the groups comprised sequences from all strains producing pI 7.6 SHV beta-lactamases and two KpI reference strains. A second group comprised sequences from all strains producing pI 7.1 LEN beta-lactamases and the two KpIII

reference strains. The third group comprised sequences from strains producing beta-lactamases different from SHV and LEN, both as determined by isoelectric focusing and by nucleotide sequencing. Two of these strains were the two KpII reference strains. None of the sequences in the third group matched closely to any sequence in public databases. Even though this group was more heterogeneous than the two other groups, we decided to assign a new beta-lactamase gene family and named it *bla*_{OKP} (other K. pneumoniae beta-lactamases).

The correspondence between chromosomal beta-lactamase gene variants and *K. pneumoniae* phylogenetic groups is illustrated in Fig. 3. The data indicate parallel evolution of *bla* and *gyrA*. All sequences belonging to the *bla*_{SHV} group were from KpI strains, as determined by *gyrA* sequencing, all *bla*_{LEN} sequences were from KpIII strains, and the *bla*_{OKP} sequences were all from KpII strains.

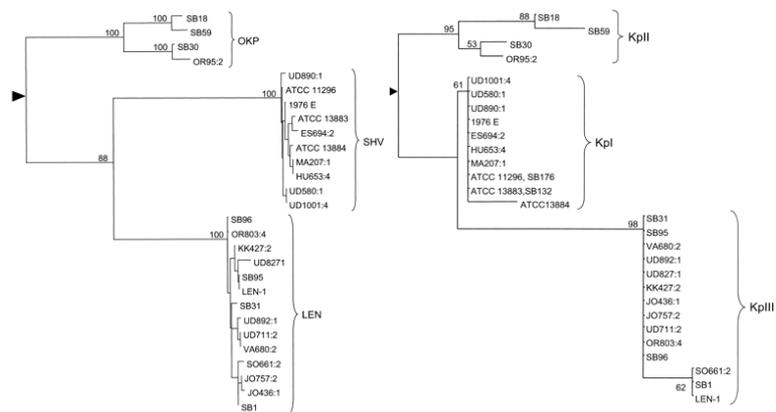


Fig. 3. Phylogenies of the *K.pneumoniae* chromosomal beta-lactamase gene (left) and the *gyrA* gene (right). The tree was obtained by the neighbor-joining method. The root was determined using the DNA sequence for beta-lactamase TEM-1, the closest known relative to the chromosomal beta-lactamase of *K. pneumoniae*. Because of the long branch leading to the TEM-1 sequence, the root position is represented by a triangle in order to increase the scale of the figure so that the relationships among *K. pneumoniae* sequences are clearly visible.

The results supported the high diversity among the *K. pneumoniae* chromosomal beta-lactamase genes seen previously (Paper I). Alignment of the 789-bp sequences from the 28 strains revealed 156 polymorphic

sites. In total, we identified 24 distinct *bla* alleles and 17 deduced amino acid sequences, including one new SHV beta-lactamase variant, seven new LEN variants, and four OKP variants. Interestingly, both strains isolated from plants produced LEN beta-lactamases. This is interesting since the beta-lactamase gene in the single full genome sequence publically available presently from a plant isolate is a *bla*_{LEN} gene (Fouts et al. 2008). However, more plant isolates need to be analyzed before any conclusions about the distribution of different beta-lactamase gene families among different *K. pneumoniae* hosts one could be drawn.

In addition to nucleotide sequencing, the 28 *K. pneumoniae* strains were subjected to MIC determinations. All strains were found to produce a typical class A group 2 broad-spectrum penicillinase inhibited by clavulanic acid, according to the Bush-Jacoby-Medeiros classification scheme (Bush et al. 1995). No ESBL activity was detected. This was in agreement with the deduced amino acid sequences, which did not reveal any substitutions associated with extended-spectrum beta-lactamase activity (Hujer et al. 2001).

The strikingly high correlation demonstrated between chromosomal beta-lactamase gene variants and *K. pneumoniae* phylogenetic groups encouraged us to investigate whether the beta-lactamase gene had been inherent to this species for a long time. In order to do this, we estimated the time since the divergence of the *K. pneumoniae* phylogenetic groups. This was done by analyzing the *mdh* gene sequences based on the molecular clock hypothesis, appreciating that DNA sequence variation cannot be perfectly explained this way. The molecular clock hypothesis states that the evolutionary rate of a gene is roughly constant among different lineages. Under the molecular clock hypothesis, sequence variation at synonymous sites is used to estimate the time since the existence of the last common ancestor. The reasons for choosing the *mdh* gene were that (i) the rate of evolution for this gene could be calibrated because nucleotide sequences of this gene were previously determined for natural populations of *E. coli* and *Salmonella enterica*, and (ii) the time since divergence between *E. coli* and *S. enterica* was previously estimated by different approaches. By using the extreme values of the various estimates of the time since divergence between *E. coli* and *S. enterica*, 30 and 140 million years, to calibrate the rate of substitution, we arrived at an estimated time since divergence between

the two most distantly related phylogenetic groups, KpI (SHV-producing strains) and KpII (LEN-producing strains), of 6 to 28 million years.

In summary, our results show that the chromosomal beta-lactamase gene has not been acquired recently by *K. pneumoniae* in response to clinical use of beta-lactam antibiotics. It has rather evolved along *K. pneumoniae* phylogenetic lineages for millions of years – bla_{SHV} along the KpI lineage, bla_{LEN} along the KpIII lineage, and bla_{OKP} along the KpII lineage. The hypothesis that clinical use of beta-lactam antibiotics would have selected for acquisition of this gene is also challenged by the finding of the same gene variant, bla_{LEN} , in both plant isolates and human clinical isolates.

7 GENETIC CONTEXTS OF BLA_{SHV} IN *K. PNEUMONIAE* CHROMOSOMES AND PLASMIDS FROM DIFFERENT GRAM-NEGATIVE BACTERIA

7.1 PAPER III

Low initial transposition frequency of chromosomal bla_{SHV} and subsequent evolution have formed the present population of acquired bla_{SHV}

Paper III started as a study of the genetic context of a *K. pneumoniae* chromosomal SHV-1 beta-lactamase gene, bla_{SHV-1} . This was done in order to investigate whether bla_{SHV-1} was located within a ≥ 5 kb transposable element as indicated by previous restriction length polymorphism analysis (Paper I, Fig. 2).

At the time, 1998, bla_{SHV} was generally regarded as a plasmid-borne gene. Evidence that the SHV-1 beta-lactamase gene existed as part of a transposon of molecular mass 9.5 megadaltons (~ 14.3 kb) in two unrelated plasmids had been published (Nugent and Hedges 1979). Results in Papers I and II, however, supported the notion of the presence of an inherent chromosomal beta-lactamase gene in *K. pneumoniae*, belonging to any of the three gene families bla_{SHV} , bla_{LEN} , and bla_{OKP} which are specific for the three phylogenetic groups of *K. pneumoniae* (Paper II, Figs. 1 and 2).

K. pneumoniae ATCC 13883^T was chosen for this part of the study. The reasons for this were that (i) this is the type strain of *K. pneumoniae*, i.e., an “old” strain, isolated in the pre-antibiotic era, which has not been under selective pressure of clinical use of beta-lactam antibiotics, and (ii) the nucleotide sequence of the chromosomal bla_{SHV-1} gene in this strain is highly similar to the sequence of the prototype bla_{SHV-1} in plasmid R974 (Paper I, Fig. 3).

This study started when the number of publically available nucleotide sequences was very low compared to today. The only publically available chromosomal *K. pneumoniae* beta-lactamase gene sequence was that of bla_{LEN-1} (Arakawa, Ohta et al. 1986). This meant that the chromosomal bla_{SHV-1} and surrounding DNA had to be cloned from *K.*

pneumoniae ATCC 13883^T before sequencing could be performed. The *bla*_{SHV-1} gene was located by Southern blot hybridization to an 8.4 kb *Eco*RI fragment, which was cloned into *E. coli* and selected for by using a cloning vector containing a kanamycin resistance gene. Transformants resistant to both ampicillin and kanamycin were selected and screened by *bla*_{SHV} PCR. A shotgun library was constructed from one *bla*_{SHV} positive recombinant plasmid. The 8.4 kb *Eco*RI insert was agarose gel purified and randomly fragmented. The fragments were modified at their ends with T4 and *Taq* polymerase and then cloned by using the cloning vector pGEM-T Easy. Clones containing inserted *K. pneumoniae* DNA were selected on agar plates containing ampicillin and IPTG/X-Gal for blue/white screening. Selected clones were sequenced using vector specific primers. After having completed the 8.4 kb sequence and not detected any genes with known function or any mobile genetic elements, the sequence was extended beyond the *Eco*RI site upstream of *bla*_{SHV-1}. This was done by inverse PCR and primer walking. The final 10.6 kb sequence contained 10 open reading frames, of which only three represented genes of known function, namely *bla*_{SHV-1}, *lacY*, and *lacZ* (Fig. 4).

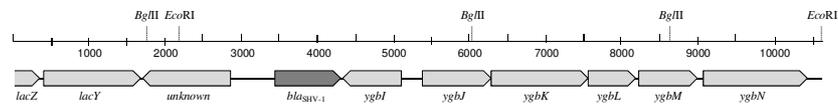


Fig. 4. Schematic representation of the genetic context of the chromosomal *bla*_{SHV-1} in *K. pneumoniae* ATCC 13883^T (adapted from Paper III, Fig. 1).

In april 2010, there were three *K. pneumoniae* complete genome sequences publically available: GenBank accession nos. CP000647 (a clinical isolate carrying *bla*_{SHV}), CP000964 (a nitrogen-fixing plant endophyte carrying *bla*_{LEN}), and AP006725 (a clinical isolate carrying *bla*_{SHV}). In all of them the chromosomal beta-lactamase gene was located in the same genetic context as *bla*_{SHV-1} in *K. pneumoniae* ATCC 13883^T. The GC content, 57%, of these chromosomes was similar in our 10.6 kb sequence, 60%. This supported the notion that the chromosomal beta-lactamase gene is inherent to *K. pneumoniae* and not part of a horizontally acquired transposon or other type of mobile genetic element.

By comparing the identified chromosomal 10.6 kb sequence of *K. pneumoniae* ATCC 13883^T to plasmid sequences publically available at the time (late 1990s), we found plasmid-located *bla*_{SHV} to be surrounded by DNA highly similar to our *K. pneumoniae* chromosomal DNA (REFS?). Therefore, in order to gain further knowledge about evolutionary relationships between chromosome- and plasmid-encoded SHV beta-lactamases, we investigated the genetic context of *bla*_{SHV} also in plasmids. This was started by performing re-sequencing of *bla*_{SHV-1} and surrounding DNA from an *E. coli* strain (BAB273) producing a pI 7.6 SHV-1 beta-lactamase. We chose to work with *E. coli* because the chromosomal beta-lactamase gene inherent to this species, *bla*_{AmpC}, is different from *bla*_{SHV} and would not cause ambiguous sequencing results. With the 10.6 kb *K. pneumoniae* ATCC 13883^T sequence available it was possible to design primers for direct sequencing of plasmid DNA. By primer walking a 4.2 kb sequence highly similar to the *K. pneumoniae* ATCC 13883^T chromosomal DNA was identified. This *bla*_{SHV}-containing sequence was flanked by IS26 elements in direct orientation. This supported the notion of a *bla*_{SHV-1} transposon in plasmids, however it was less than half the size estimated by Nugent and Hedges (Nugent and Hedges 1979). The IS26 element located upstream of *bla*_{SHV-1} interrupted *lacY*, and the downstream IS26 element interrupted the gene next to *bla*_{SHV}, *ygbI*.

The finding that DNA highly similar to the chromosome of *K. pneumoniae* surrounded *bla*_{SHV} in plasmids also added evidence to our suggestion, in Paper I, that an allelic variant of the chromosomal beta-lactamase gene in *K. pneumoniae* is the ancestor of *bla*_{SHV} genes carried and spread by plasmids.

Among the plasmid sequences publically available at the time (late 1990s and early 2000s) only a few contained both *bla*_{SHV} and IS26, and none of the sequences contained more than one IS26 element (Naas, Philippon et al. 1999). The presence of an IS26 element at both ends of the *bla*_{SHV}-containing sequence suggested IS26-mediated mobilization of a chromosomal *bla*_{SHV}-containing fragment from *K. pneumoniae* to plasmid, and subsequent mobilization between plasmids. This idea had been presented earlier, and is now regarded as the most likely evolutionary scenario (Ford and Avison 2004), (Miriagou, Carattoli et al. 2005), (Garza-Ramos, Davila et al. 2009). Later, chromosomal origin of other

beta-lactamase genes have been demonstrated, e.g., the ancestor of the currently widespread *bla*_{CTX-M} genes originates from *Kluyvera ascorbata* (Golebiewski, Kern-Zdanowicz et al. 2007). However, these genes are mobilized by other genetic mobile elements than IS26.

To further analyze the genetic context of *bla*_{SHV} in plasmids, 11 additional SHV beta-lactamase-producing *E. coli* strains were included in the study. These strains were, like *E. coli* BAB273, from the culture collection at the section for antimicrobial resistance and infection control at SMI. They were isolated from patients in different parts of Sweden 1996 to 2003. There were no epidemiological links between the patients as judged by information about time and place of isolation. One of the strains produced a pI 7.6 SHV-1 beta-lactamase and the others produced different SHV ESBLs conferring resistance, inhibited by clavulanic acid, to cefotaxime and ceftazidime. Two of the ESBLs were pI 7.6 enzymes and six were pI 8.2 enzymes.

PCRs developed in order to amplify possible IS26-flanked *bla*_{SHV}-containing sequences were positive for all 11 *E. coli* strains analyzed. In two strains the sequence had the same length as in *E. coli* BAB273, in others it was of different lengths. Sequence comparisons revealed that all sequences were highly similar to *K. pneumoniae* chromosomal DNA. The sequences formed two groups. In each group the flanking IS26 elements were located at specific distances from *bla*_{SHV}. In total, five distinct junctions between IS26 and *bla*_{SHV}-containing sequence highly similar to chromosomal *K. pneumoniae* DNA were identified. Two of these were located upstream of *bla*_{SHV} and three downstream of the beta-lactamase gene.

Later, when the number of sequences publically available had increased enormously, 33 sequences containing both *bla*_{SHV} and surrounding DNA were retrieved from the GenBank nucleotide sequence database nt/nr by performing a Blastn using our 10.6 kb *K. pneumoniae* ATCC 13883^T sequence as query (Paper III, Table 1). These sequences were compared to our *K. pneumoniae* chromosomal and *E. coli* plasmid sequences. The comparison demonstrated high diversity among chromosomal *K. pneumoniae* sequences, and low diversity among the plasmid sequences. The published plasmid sequences were from plasmids carried by different gram-negative hosts, but each of the sequences was highly similar to one of the two groups identified for the

sequences of the 11 *E. coli* strains analyzed (Paper III, Table 2). Among the sequences that contained IS26, some had the same IS26-junction(s) as seen in our *E. coli* sequences, and some had other junctions. This resulted in the overall identification of nine distinct IS26-junctions, which in specific combinations characterize seven lengths of IS26-flanked *bla*_{SHV}-containing plasmid sequences (Table 5).

TABLE 5. Characteristics of the seven IS26-flanked *bla*_{SHV}-containing sequences identified in plasmids carried by different pathogenic gram-negative bacteria

IS26-junctions ^a	Length (kb)	Sequence group ^b	Plasmid host(s) ^c
J1 and J9	8.0	PI	<i>E. coli</i> , <i>E. cloacae</i> , <i>S. Typhimurium</i> , <i>Y. pestis</i>
J2 and J9	6.7	PI	<i>K. pneumoniae</i>
J1 and J6	4.2	PI	<i>E. coli</i>
JX and J4	≥2.4	PI	<i>K. pneumoniae</i>
J3 and J8	4.5	PII	<i>K. pneumoniae</i> , <i>E. coli</i> , <i>E. cloacae</i>
J3 and J7	1.9	PII	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. Typhimurium</i>
J3 and J5	1.4	PII	<i>E. coli</i>

^a Each junction between IS26 and the *bla*_{SHV}-containing sequence highly similar to *K. pneumoniae* chromosomal DNA was numbered on the basis of its position relative to the 10.6 kb fragment of *K. pneumoniae* ATCC 13883T chromosomal DNA sequence; J1 closest to the 5'-end of the fragment and J9 closest to the 3'-end (Paper III, Fig. 1). Detailed information on which part of the *K. pneumoniae* chromosome each sequence length corresponds to can be seen in Paper III (Table 1).

^b The sequence groups are based on SNP analysis of 2.8 kb sequences containing *bla*_{SHV}, *ygbI*, and *ygbJ* (Paper III, Table 2).

The finding of two plasmid sequence groups was in agreement with data published later by Ford and Avison (Ford and Avison 2004). They used bioinformatic approaches to study publically available full-length *bla*_{SHV} gene sequences, and constructed an evolutionary tree in which they identified two main branches. Both branches derived from a *bla*_{SHV-1} variant. They concluded that the *bla*_{SHV} gene had been mobilized at least twice, and that the mobilization events had been catalyzed by IS26.

In summary, our results support the idea, presented in Paper I, that the *bla*_{SHV} variants seen among plasmids originate from the chromosome of one or more strains closely related to *K. pneumoniae* ATCC 13883^T. They also support the notion of an evolutionary scenario involving IS26-mediated mobilization of *bla*_{SHV}. However, mobilization from chromosome to plasmid seems to be a rare event. The ongoing spread of *bla*_{SHV} genes occurs as IS26-mediated mobilization of *bla*_{SHV}-containing DNA fragments, of different lengths, representing two

evolutionary lineages. These IS26-flanked *bla*_{SHV}-containing fragments are carried by a wide range of plasmids found in many pathogenic gram-negative bacterial species. Taken together, this implies that chromosomal *K. pneumoniae* *bla*_{SHV} genes are not part of the gene pool contributing to the current spread of SHV beta-lactamase producing strains.

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