3

The Natural Antibiotic Resistances of the Enterobacteriaceae *Rahnella* and *Ewingella*

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1. Introduction

The antibiotic resistance genes present in clinical isolates are usually acquired and located on mobile elements allowing their horizontal transfer to other strains or even across bacterial species. Consequently, resistance genes with 100% sequence identity may be found in otherwise unrelated genera while the occurrence of such an acquired resistance within a certain species is highly variable.

In contrast, a number of bacteria are naturally resistant against some antibiotics. The molecular basis for natural resistance may be a general factor like the lack of the targeted pathway, a variant of the targeted molecule that is not inhibited by the antibiotic or a membrane limiting entry of the antibiotic into the cell. In addition natural resistance may also be mediated by a resistance gene belonging to the cell's core genes. Such resistance genes are vertically inherited, shared by (nearly) all isolates of a species and co-evolve with their hosts. They are often encoded by the chromosome, are usually immobile and their expression level is tightly regulated or very low. The establishment of such a resistance requires a long lasting, usually mild selection pressure as it may be present in the soil, which contains many microorganisms producing antibiotics. Examples for this type of natural resistance are the chromosomally encoded β-lactamases found in several species of the Enterobacteriaceae (Naas et al., 2008), many of them colonising plants and soil.

Although these environmental microorganisms pose a low risk to human health, concerns about the spread of their antibiotic resistance genes to pathogens have arisen. Their resistance genes are usually non-mobile, but inclusion into mobile genetic elements may allow the spread to unrelated bacteria. In the last two decades the CTX-M type enzymes have become the most prevalent extended-spectrum β-lactamases (EBSLs) in pathogenic Enterobacteriaceae (Canton & Coque, 2006). The CTX-M enzymes are believed to originate from *Klyvera ascorbata* and *Klyvera georgiana* chromosomal β-lactamases (Olson et al., 2005; Rodriguez et al., 2004). The inclusion of these genes in integrons located on large conjugative plasmids has likely facilitated their spread among the Enterobacteriaceae. Such plasmids contain frequently multiple resistance genes, which might have further enhanced spread of the CTX-M genes in microbial communities by co-selection (Canton & Coque, 2006). Once established in pathogens the spectrum of the resistance genes may be increased by point mutations further impeding treatment of infections with antibiotics. Thus

improved understanding of natural resistance, conditions favouring transfer of resistance genes to pathogens and the underlying molecular mechanisms are important areas of research.

Rahnella and *Ewingella*, two closely related genera of the Enterobacteriaceae, are naturally resistant to several β-lactam antibiotics. *Rahnella* is widespread in nature and routinely present in the daily human diet but also *Ewingella* may be present at high titers in some kinds of food. Both microorganisms have been infrequently isolated from clinical specimens. Here the biology, natural habitats, clinical significance and antibiotic susceptibility patterns of *Ewingella* and *Rahnella* will be addressed. Novel results about their resistance genes will be presented and the evolution of these genes and the potential for their transfer to other bacteria will be discussed.

2. Biology, clinical significance and antibiotic resistances of *Rahnella* and *Ewingella*

In 1976 a new class of Enterobacteriaceae was defined during a numerical taxonomy study and provisionally named 'group H2' (Gavini et al., 1976). Based on DNA relatedness studies this group was later proposed as a new species, *Rahnella aquatilis* (Izard et al., 1979). In the following years strains belonging to this novel genus were infrequently isolated from water and clinical specimens and *Rahnella* was thought to be a rare microorganism (Farmer et al., 1985) until it was found to be frequent in plant and soil specimens. Also *Ewingella* was recognised as a separate group of the Enterobacteriaceae in a phenotypical study, which was subsequently confirmed by DNA-DNA hybridisation experiments (Grimont et al., 1983). Based on current reports *Ewingella* is believed to be a rare member of the Enterobacteriaceae (Brenner & Farmer 2005) but some studies indicate that it might be common in some ecological niches. Investigations of clinical isolates revealed that *Rahnella* and *Ewingella* are resistant to several antibiotics, mainly β–lactams. The susceptibility patterns suggested the presence of an extended spectrum Ambler class A β-lactamase (ESBL) in *Rahnella* (Stock et al., 2000), which could be confirmed by cloning and sequencing of the resistance gene (Bellais et al., 2001). The susceptibility pattern and detection of the enzyme by SDS-PAGE/nitrocefin staining suggested an Ambler class C β-lactamase (AmpC) for *Ewingella* (Stock et al., 2003). Here we report for the first time a DNA sequence-based phylogenetic analysis confirming that the *Ewingella* β-lactamase belongs to the AmpC class.

2.1 Biology, habitat and possible applications of *Rahnella* and *Ewingella*

The genus *Rahnella* comprises three genomospecies, *Rahnella aquatilis* (= genomospecies 1), *Rahnella* genomospecies 2 and *Rahnella* genomospecies 3 (Brenner et al., 1998), while the genus *Ewingella* consists of only one species: *Ewingella americana*. Based on phenotypical tests two biogroups of *Ewingella americana* have been defined, which show differences in L-rhamnose and D-xylose fermentation (Grimont et al., 1983). Strains belonging to *Rahnella* and *Ewingella* have no special nutritional requirements and can use a number of carbon sources. They are able to grow in the temperature range from close to 0°C to approximately 40°C, although many strains show a reduced biochemical activity at elevated temperatures (Brenner & Farmer 2005; Brenner et al., 1998; Davis & Eyles, 1992; Jensen et al., 2001; McNeil et al., 1987).

Rahnella is widely distributed and has been isolated from many types of samples. It is frequently found in the rhizosphere and tightly associated with roots and tubers of plants (Berge et al., 1991; Heulin et al., 1994; Jafra et al., 2009; Rozhon et al., 2010) but is also present on other parts of plants including leaves (Hamilton-Miller & Shah, 2001; Hashidoko et al., 2002), fruits (Lindow et al., 1998) and seeds (Cankar et al., 2005; Iimura & Hosono, 1996). Other sources are water (Brenner et al., 1998; Gavini et al., 1976; Niemi et al., 2001), soil (Martinez et al., 2007) and the intestine of snails, slugs (Brenner et al., 1998) and even American mastodon remains (Rhodes et al., 1998). Recently, *Rahnella* was also found at a high frequency in the gut of ghost moths (Yu et al., 2008) and to be associated with larvae and adults of the mountain pine beetle (Winder et al., 2010). *Rahnella* is frequently present in the human diet and has been isolated from different types of food including vegetables (Hamilton-Miller & Shah, 2001; Raphael et al., 2011; Rozhon et al., 2010; Ruimy et al., 2010a), sprouts (Cobo Molinos et al., 2009), fruits (Rozhon et al., 2006), meat (Brightwell et al., 2007; Lindberg et al., 1998) and beverages (Hamze et al., 1991; Jensen et al., 2001). In contrast to its wide distribution in nature *Rahnella* is rarely isolated from clinical specimens.

Ewingella has also been isolated from vegetables (Hamilton-Miller & Shah, 2001) and vacuum-packaged meat (Brightwell et al., 2007), but seems to be significantly less frequent than *Rahnella* in such samples. In contrast, *Ewingella* is very common on mushrooms including button mushroom, shiitake and oyster mushroom (Reyes et al., 2004). Importantly, *Ewingella* is the causative agent of a browning disorder of button mushroom called 'internal stipe necrosis' (Inglis & Peberdy, 1996), which causes significant economic loss. In addition, *Ewingella* has also been isolated from molluscs (Müller et al., 1995). Clinical specimens tested positive for *Ewingella* were mainly blood and swabs from the respiratory tract and wounds.

Rahnella and *Ewingella* have some interesting properties for agronomic and industrial applications. Both seem to promote plant growth and *Rahnella* may be useful as antagonist for controlling plant pathogens including *Erwinia amylovora*, causing fire blight of pear and apple trees (Laux et al., 2002), and *Xanthomonas campestris*, the causative agent of black rot (El-Hendawy et al., 2005). In addition, *Rahnella* might improve the supply of plants with nutrients like phosphate (Kim et al., 1997) and it is able to fix nitrogen (Heulin et al., 1994). The polysaccharides levan and lactan produced by different strains of *Rahnella* have interesting properties for industrial processes (Kim et al., 2003; Matsuyama et al., 1999; Pintado et al., 1999; Seo et al., 2002). The high uranium(VI) resistance of *Rahnella* and its ability to bind this toxic heavy metal is currently intensively investigated and its potential for bioremediation is studied (Beazley et al., 2007; Geissler et al., 2009; Martinez et al., 2007). Because of the increasing interest a project for sequencing of the *Rahnella* genome was launched and recently finished. The sequence of environmental strain *Rahnella aquatilis* Y9602 is available from the genbank database (www.ncbi.nlm.nih.gov) under accession number NC_015061.

2.2 Clinical significance

Rahnella and *Ewingella* are only occasionally isolated from clinical specimens and the clinical significance of both microorganisms is still under debate. Both are believed to be opportunistic pathogens. The pathogenic potential of *Rahnella* seems to be relatively low while a few fatal outcomes of infections caused by *Ewingella* have been reported.

2.2.1 Clinical significance of *Rahnella*

Several reports describe the isolation of *Rahnella* in a clinical context (Table 1). However, in some cases the clinical significance is difficult to assess particularly because many patients had some underlying conditions including haematologic and solid organ malignancy, diabetes and AIDS or had undergone surgery. The age of the patients ranged from 11 months to 78 years and an, although statistically insignificant, male predominance has been recognised among them (Gaitán & Bronze, 2010). Typical sites of isolation were blood, wounds and urine. Interestingly, a significant number of patients developed symptoms during hospitalisation suggesting nosocomial infections.

The first description of *Rahnella* in a clinical context dates back to 1985, where it was isolated from a burn wound (Farmer et al., 1985). In another case *Rahnella* was isolated from a surgical wound that had persisted for more than eight months and was repeatedly tested negative for bacteria before a purulent exudate appeared. At that time pure cultures of *Rahnella* could be isolated from the wound exudate (Maraki et al., 1994). Since *Rahnella* is easy to cultivate and previous efforts to detect bacteria in the wound were negative it seems most likely that the wound was infected recently before the exudate appeared, for instance during the daily wound cleansing procedure. In a further case *Rahnella* was isolated from a diabetes mellitus associated foot wound. Although the infection reacted well to treatment with ampicillin-sublactam the toe and the second digit of the foot had to be amputated because of severe necrosis. This course of disease belongs to the most severe described for an infection with *Rahnella*. However, the ulceration of the wound had begun two month before any medical treatment was started and a co-infection with *Candida sp.* was diagnosed.

While, in a clinical context, *Rahnella* was first isolated from a wound swab, its most frequent site of isolation was blood. *Rahnella* bacteraemia was associated with fever and in two cases with septic shock (Chang et al., 1999; Gaitán & Bronze, 2010). Most patients showed *Rahnella* bacteraemia during hospitalisation (9 of 15 cases) and venous catheters, surgery and drug abuse seem to pose risk factors for infection with this bacterium (Funke & Rosner, 1995; Gaitán & Bronze, 2010; Hoppe et al., 1993; Oh & Tay, 1995). In two epidemiologically related cases a parenteral nutrition fluid was identified as the most probable source of *Rahnella* (Caroff et al., 1998). Both cases appeared in the same hospital within three days and the bacterial strains isolated from the blood of both patients showed identical biochemical profiles and antibiograms and shared the same macrorestriction and ribotyping profiles. Also other patients who had received the same batch of the parenteral nutrition fluid experienced episodes of shivers but blood cultures were not taken impeding further analysis (Caroff et al., 1998). In one very unusual case a contaminated intravenous infusion fluid that a patient had self-administrated could be identified as the source of *Rahnella* (Chang et al., 1999). Thus in a number of cases *Rahnella* cells were directly introduced into the blood circulation. Under certain circumstances *Rahnella* may also be able to spread from the urinary tract to the blood system. Blood cultures of a febrile 76-year old man complaining of nausea and vomiting grew *Rahnella*. The patient had a history of a benign prostatic hypertrophy and the analysis of his urine revealed "many" bacteria. Because of these results and the underlying conditions pyelonephritis was suggested as a possible source of the patient's bacteraemia (Tash, 2005). Since the bacteria isolated from blood and urine of this patient were not compared by biochemical and molecular methods a causal link between the urinary tract infection and bacteraemia remains speculative. With respect to that it is important to note that *Rahnella* was isolated from urine in some other cases but no signs for bacteraemia were reported (Alballaa et al., 1992; Domann et al., 2003; O'Hara et al., 1998).

Case	Year, country	Age, sex	Signs and symptoms	Site	Underlying condition(s)	Treatment	Outcome	Reference
1	1985[a], USA	NA	NA	Burn wound	Burn	NA	NA	(Farmer et al., 1985)
2	1986, USA	37 y, M	Cough, fever, night sweats, diarrhoea	Bronchial washings	AIDS; co-infection with *Cryptococcus neoformans*	Ampicillin, gentamycin	Cure	(Harrell et al., 1989)
3	1987, Belgium	79 y, M	Fever, expectoration	Sputum, bronchial aspirate	Chronic lymphocytic leukaemia emphysema, bronchopulmonary infection with *Pneumococcus*	Trimethoprim-sulfamethoxazole	Cure	(Christiaens et al., 1987)
4	1988, France	42 y, F	Septicaemia, leukaemic relapse	Blood	Acute lymphocytic leukaemia, diabetes mellitus, bronchial asthma, Hickman catheter	Vancomycin, ceftazidime	NA	(Goubau et al., 1988)
5	1991, Saudi Arabia	40 y, M	Dysuria	Urine	Renal transplant (status post)	Amoxicillin; ciprofloxacin	Cure	(Alballaa et al., 1992)
6	1992, Greece	63 y, F	Purulent exudate	Surgical wound	Osteoporosis, alcoholism, operation at the left knee	Trimethoprim-sulfamethoxazole	Cure	(Maraki et al., 1994)
7	1992, Germany	7 y, M	Fever (39.5°C)	Blood	Bone marrow transplant recipient; Hickman catheter	Gentamycin, azlocillin, flucloxacllin; amikacin, ceftriaxone, vancomycin	Cure	(Hoppe et al., 1993)
8	1994[a], Italy	59 y, F	Fever	Blood	Chronic renal failure, parenteral nutrition via a Hickman catheter	Ciprofloxacin	Cure	(Caraccio et al., 1994)
9	1994, Switzerland	21 y, M	Fever (39°C)	Blood	AIDS, positive for HBV, HCV and HDV antibodies, recent infection with *Staphylococcus aureus*, intravenous drug abuse	Ciprofloxacin	Cure	(Funke & Rosner, 1995)
10	1995[a], Singapore	48 y, M	Fever (38.2°C)	Blood	Diabetes mellitus (for 2 y), pulmonary tuberculosis, appendicular abscess	Ampicillin, gentamycin, amoxicillin-clavulanate, gentamycin	Cure	(Oh & Tay, 1995)
11	1995[a], Singapore	57 y, M	Fever	Blood	Laryngeal carcinoma, total laryngectomy	Metronidazole, ceftriaxone; gentamycin	Cure	(Oh & Tay, 1995)
12	1996[a], Spain	2 y, F	Acute gastroenteritis	Faeces	None	None	Cure	(Reina & Lopez, 1996)
13	1996[a], Spain	2 y, M	Acute gastroenteritis	Faeces	AIDS	None	Cure	(Reina & Lopez, 1996)

Case	Year, country	Age, sex	Signs and symptoms	Site	Underlying condition(s)	Treatment
14	1996[a], Japan	11 m, F	Fever (39.7°C), cough	Blood	Congenital heart disease	Cefpodoxime-prox cefotaxime; cefazo netilmicin, ceftazic
15	1997, France	32 y, F	Fever (>38°C)	Blood	Ingestion of a caustic product, parenteral nutrition via a catheter	Removal of the cat
16	1997, France	61 y, M	Fever (40°C)	Blood	Relapse from a renal carcinoma (status post), parenteral nutrition via a catheter	Ticarcillin-clavular vancomycine
17	1998[a,b], Japan	NA	NA	Urine	Chronic urinary tract infection	NA
18	1999, Korea	26 y, M	Fever (38.2°C), septic shock	Blood	Contaminated intravenous fluid; healthy individual	Ceftriaxone, imipe
19	1999[a], Tunisia	65 y, F	Fever (38.5°C), ketosis	Blood	Diabetes mellitus for 5 y	Cefotaxime, trimethoprim-sulfamethoxazole
20	2000, USA	46 y, M	Fever	Blood	B-cell lymphoblastic leukaemia, immunosuppressive medication, Hickman catheter	Piperacillin-tazoba gentamycin
21	2000, Spain	63 y, M	Fever (37.8°C), excessive exudate	Trache-ostomy exudate	Laryngeal carcinoma (status post)	Amoxicillin-clavul acid; cefotaxime, amikacin
22	2003[a], NA	NA, F	NA	Urine	Co-infection with *Candiada albicans*	None
23	2004[a], USA	76 y, M	Fever (39.8°C)	Blood	Benign prostatic hypertrophy, bacteria in urine	Tracheostomy tub Levofloxacin
24	2009[a], Turkey	57 y, F	Ulcerated foot wound	Wound	Diabetes mellitus for 20 years, co-infection with *Candida* sp.	Ampicillin-sublact Amputation of a t
25	2009, Italy	78 y, M	Fever, sepsis	Blood	hospitalised at an intensive care unit, co-infection with *Candida famata* and *Pantoea agglomerans*[c]	Meropenem
26	2011, USA	27 y, F	Septic shock, fever (38.1°C)	Blood	Sickle cell disease, central venous catheter	Ciprofloxacin, rem of the catheter

Table 1. Infections caused by *Rahnella*. All cases we could find in the literature are included. [a] Year of not available); [b] The isolates were obtained in the 1990s; [c] *Pantoea agglomerans* is considered as the rea

Rahnella was also isolated from the faeces of two children with acute diarrhoea. In both cases typical enteropathogenic bacteria, parasites and viruses could not be detected. However, the detection of *Rahnella* in the faeces of patients with diarrhoea is not a sufficient reason for the conclusion that this microorganism is the true cause of the infectious process (Reina & Lopez, 1996). It seems indeed unlikely that *Rahnella* is an enteropathogen since this organism is frequently present in food, particularly vegetables which are frequently eaten raw, while the isolation of *Rahnella* from faeces from patients suffering acute gastroenteritis seems to be a rare exception.

Infections with *Rahnella* reacted very well to treatment with antibiotics and most patients recovered rapidly, though even many of them were immunocompromised. Some patients recovered even without antibiotic treatment (Caroff et al., 1998; Reina & Lopez, 1996). Importantly, no deaths were reported as outcome of an infection with *Rahnella*. These data and the fact that *Rahnella* is a frequent microorganism routinely present in the human diet suggest that it has only a slight pathogenic capacity and its ability to infect humans may be highly dependent on their immunological status.

Currently few data about the pathogenic capacities of the three genomospecies of *Rahnella* are available. The routinely used phenotypic tests allow identification of *Rahnella* only at the genus level. Thus the genomospecies of the isolates of the cases summarised in Table 1 is unknown. A study using DNA-DNA hybridisation revealed that three clinical isolates belonged to *Rahnella aquatilis* (= genomospecies 1) and three were identified as *Rahnella* genomospecies 2 (Brenner et al., 1998) indicating that both genomospecies may act as opportunistic pathogens. However, a study including more strains is highly demanded to assess any potential differences of the pathogenic potential of the *Rahnella* genomospecies.

2.2.2 Clinical significance of *Ewingella americana*

Ewingella americana has been isolated from a variety of clinical specimens, particularly blood and wound swabs and less frequently from sputum (Brenner & Farmer 2005). Typical underlying conditions were surgeries, injuries from accidents, drug abuse and renal failure (Table 2). Some patients had diabetes, received immunosuppressive therapy, were HIV positive or suffered from other chronic infections. However, in contrast to infections with *Rahnella*, a significant number of patients were fully immunocompetent.

Most patients had undergone surgery prior development of bacteraemia, suggesting nosocomial infections. Pien and Bruce (1986) described a nosocomial outbreak of *Ewingella* bacteraemia. Six cases of *Ewingella* bacteraemia appeared in an intensive care unit of a hospital within six weeks. All infected patients had high fever or leukocytosis and had undergone either cardiovascular or peripheral vascular surgery. A careful environmental culturing study identified a contaminated ice bath used to cool syringes for cardiac output determinations as most likely source for the bacteria. *Ewingella americana* was cultured from the bath and its removal from the intensive care unit terminated the outbreak (Pien & Bruce, 1986). In another hospital *Ewingella americana* was diagnosed in blood drawn from 20 patients (Gardner et al., 1985). None of the patients had symptoms typical for *Ewingella americana* sepsis. An environmental investigation revealed that the bacteria were present in a citric buffer anticoagulant used to fill coagulation tubes. Review of blood drawing procedures showed that the non-sterile coagulation tubes were frequently filled first

Case	Year, country	Age, sex	Signs and symptoms	Site	Underlying condition(s)	Treatment	Outcome	Reference
1	1982-1983, USA	55 y, F	Postoperative fever, sepsis	Blood	Aortoiliac graft bypass, aorta occlusion, diabetes	Ampicillin, carbenicillin, gentamicin	Cure	(Pien & Bruce, 1986)
2	1982-1983, USA	57 y, M	Postoperative fever	Blood	Ventricular aneurysmetomy, lower extremity thrombectomy	Cefotaxime, gentamicin, mezlocillin	Cure	(Pien & Bruce, 1986)
3	1982-1983, USA	58 y, M	Postoperative fever	Blood	Coronary artery bypass surgery	Gentamicin, mezlocillin, trimethoprim-sulfamethoxazole	Cure	(Pien & Bruce, 1986)
4	1982-1983, USA	54 y, F	Postoperative fever	Blood	Aorta-iliac artery bypass	Gentamicin, trimethoprim-sulfamethoxazole, doxycycline	Cure	(Pien & Bruce, 1986)
5	1983[a], USA	41 y, M	Postoperative fever (39.2°C)	Blood	Bypass surgery; atherosclerosis, diabetes mellitus; intravascular catheters; co-infection with *Pseudomonas* sp.	Gentamicin, trimethoprim-sulfamethoxazole	Cure	(Pien et al., 1983)
6	1985 , South Africa	46 y, M	Wound (traffic accident)	Wound swab	Wounds originating from a traffic accident; co-infection with *Staphylococcus aureus*	None	Cure	(Bear et al., 1986)
7	1989, Germany	30 y, F	adhesive eyelids, itching	Conjuncti-vae (swab)	None	Amoxicillin-clavulanate	Cure	(Heizmann & Michel, 1991)
8	1991, Belgium	75 y, M	Cholecystis, fever (39.4°C)	Blood	Surgery of the gallbladder; also *Pseudomonas aeruginosa*, *Candida albicans* and *Serratia marcescens* were isolated from the patient	Temocillin	Cure	(DeVreese et al., 1992)
9	1991[a], Spain	31 y, M	Balanitis	Penile exudate	HIV, intravenous drug abuse, several opportunistic infections	Tobramycin	Cure	(Sanmartin Jimenez et al., 1991)
10	1995[a], Spain	18 m, M	Acute gastroenteritis	Faeces	None	None	Cure	(Reina et al., 1995)
11	1999[a], Greece	70 y, F	Peritonitis, fever (37.4°C)	Peritoneal dialysate	End-stage renal disease , ambulatory dialysis for 5 years	Amikacin, vancomycin	Cure	(Kati et al., 1999)

Case	Year, country	Age, sex	Signs and symptoms	Site	Underlying condition(s)	Treatment
12	1999, France	38 y, M	Fever (39°C)	Blood	AIDS, intravenous drug abuse (a syringe used was rinsed with water from a fountain), co-infection with *Candida sp.*	Ceftriaxone, amik
13	2000, Belgium	57 y, F	Fever (38.8°C)	Blood	Peripheral blood progenitor cell transplantation, treatment with cyclosporine A; Hickman catheter	Removal of the ca
14	2000[a], Brasilia	38 y, F	Kerato-conjunctivitis	Conjuncti-vae (swab)	Soft contact lens	Ciprofloxacin
15	2003[a], Germany	74 y, F	Waterhouse–Friderichsen syndrome	Blood from heart and spleen	Pain in the left leg; otherwise healthy	Tramadol (for trea of pain)
16	2004[a], Greece	72 y, M	Fever (38.5°C), diffuse abdominal pain	Peritoneal effluent	end-stage renal failure, dialysis for 3 years	Ceftazidime, tobra
17	2003[a], Korea	35 y, M	Pneumonia, fever (38.2°C)	Sputum	Chronic renal failure for 7 y; rejection of the transplanted kidney; coinfection with alpha-haemolytic streptococci	Ceftriaxone, isepa
19	2007[a], USA	77 y, F	Shortness in breath	Sputum	Infection with *Mycobacterium tuberculosis* and *M. avium* (status post); Cohen's disease	Trimethoprime-sulfamethoxazole
20	2007, Saudi Arabia	30 y, M	Pneumonia	Tracheal aspirate	Multiple severe injuries from a traffic accident, coma, contusion on the right upper lung, multiple organ failure	No treatment with antibiotics is desc

Table 2. Infections caused by *Ewingella*. All cases we could find in the literature are included. [a] Year o not available).

allowing contamination of the subsequently filled culture tubes (McNeil et al., 1985). At least some of the patients received inappropriate, unnecessary antimicrobial therapy, incurring the risk of adverse drug reactions and the selection of drug-resistant bacteria (McNeil et al., 1987).

A fatal case of Waterhouse–Friderichsen syndrome was associated with an *Ewingella* infection of a previously healthy 74-year-old women (Tsokos, 2003). She experienced dragging pain in her left leg. Since the physical examination was unremarkable except for restricted mobility caused by the painful leg and her temperature was normal, just an analgetic was administered and bed rest ordered. On the next morning she was found dead in her bed. An autopsy revealed intraparenchymal haemorrhages in both adrenal glands, the heart showed granulocytic infiltration, clots were present in the larger arterial vessels and her brain and lungs were oedematous. *Ewingella americana* could be isolated from heart and spleen blood obtained during autopsy. In agreement with a suspected sepsis a highly increased level of procalcitonin was measured. Death was attributed to acute adrenal insufficiency due to Waterhouse–Friderichsen syndrome caused by *Ewingella americana* (Tsokos, 2003). In a second case the death of a 30-year-old man was associated with pneumonia caused by *Ewingella americana* (Bukhari et al., 2008). In this case the patient was admitted deeply comatose with multiple severe injuries caused by a road traffic accident to hospital. His brain showed oedema, intercerebral haemorrhage in basal ganglia to the right thalamus and subarachnoid haemorrhage along with the fracture of the frontal bone. The upper part of his right lung showed contusion. *Ewingella americana* was identified in his tracheal aspirate but not from any other sample of the patient. The isolated strain exhibited multiple antibiotic resistances but it was not reported whether the patient received any antibiotic treatment. On the eighth day of admission he went to a stage of multiple organ failure and died. It was hypothesised that the cause of death may be pneumonia associated with brain damage (Bukhari et al., 2008). However, because of the underlying conditions it is difficult to rate whether the infection with *Ewingella* was indeed the cause of death. Only two other cases of respiratory infection caused by *Ewingella* have been reported. In both cases the patients recovered quickly after treatment with antibiotics. However, it is important to note that in one of these cases the isolated strain was multidrug resistant (Pound et al., 2007).

In two cases *Ewingella* was associated with eye infection (Da Costa et al., 2000; Heizmann & Michel, 1991). Swabs of the conjunctivae grew the microorganism. Symptoms were keratoconjunctivitis, adhesive eyelids, itching and impaired secretion of tears. In both cases the infection reacted well to antibiotic treatment and the symptoms were relieved in a few days. One report describes also the isolation of *Ewingella* from faeces of a patient with diarrhoea. However, like in the cases of isolation of *Rahnella* from faeces, the clinical significance of this finding is unclear. Since *Ewingella* may be present on some kinds of food, isolated bacteria may originate from the ingested food and be unrelated to diarrhoea. Studies on the frequency of *Ewingella* in the human diet and additional case reports are necessary to rate the enteropathogenic potential of this microorganism.

Taken together these reports suggest that *Ewingella* has a higher pathogenic capacity than *Rahnella*. Several cases of infection in immunocompetent patients were reported. *Ewingella* may also cause infections with fatal outcome. Furthermore, while all *Rahnella* strains isolated so far are susceptible to most antibiotics, two multiple drug resistant isolates of *Ewingella* have been reported. The origin of these resistances, their molecular basis and capacity to spread to other genera are intriguing questions to be addressed in the future.

2.3 Identification of *Rahnella* and *Ewingella*

Reliable identification of strains is crucial for determining appropriate treatments of infections, hygiene monitoring in medical centres and industry and for basic research studies investigating the biology and ecology of microorganisms. In the past *Rahnella* strains were often identified as *Enterobacter agglomerans*, which may also explain that *Rahnella* was thought to be a rare genus while it is now considered as a relatively frequent bacterium.

Rahnella and *Ewingella* can be isolated using media not inhibitory for Enterobacteriaceae such as MacConkey agar or Bromothymol blue lactose agar. Levine EMB agar is especially suitable for *Rahnella*, which forms dark colonies on this medium (Rozhon et al., 2010). *Ewingella* was successfully isolated from mushrooms using VRBG agar (Reyes et al., 2004) or LB agar plates. The latter were anaerobically incubated to suppress growth of *Pseudomonas* (Inglis & Peberdy, 1996). Since a single phenotypic test allowing identification of *Rahnella* or *Ewingella* is lacking, a complete set of biochemical tests is necessary for identification. *Rahnella* is often described to be phenylalanine deaminase positive, which is a very rare characteristic among the Enterobacteriaceae, and to be motile at 25°C but not at 37°C. However, it must be emphasised that *Rahnella* shows only a very weak positive reaction for phenylalanine deaminase and some isolates react negative. Similarly, some strains are also immotile at 25°C. Thus the results of these two tests should be interpreted with care. It is important to note that the three *Rahnella* genomospecies can not be differentiated by biochemical tests (Brenner et al., 1998). Nevertheless, in many reports strains are claimed to be identified as '*Rahnella aquatilis*' although only phenotypic tests were performed. Such classifications should be evaluated very critically. The three *Rahnella* genomospecies were originally identified by DNA-DNA hybridisation experiments (Brenner et al., 1998). With the rapid development of molecular techniques in the last decades DNA sequencing of housekeeping genes is now the method of choice for identification of *Rahnella* at the genomospecies level and for confirmation of the identification of *Ewingella*. For sequencing

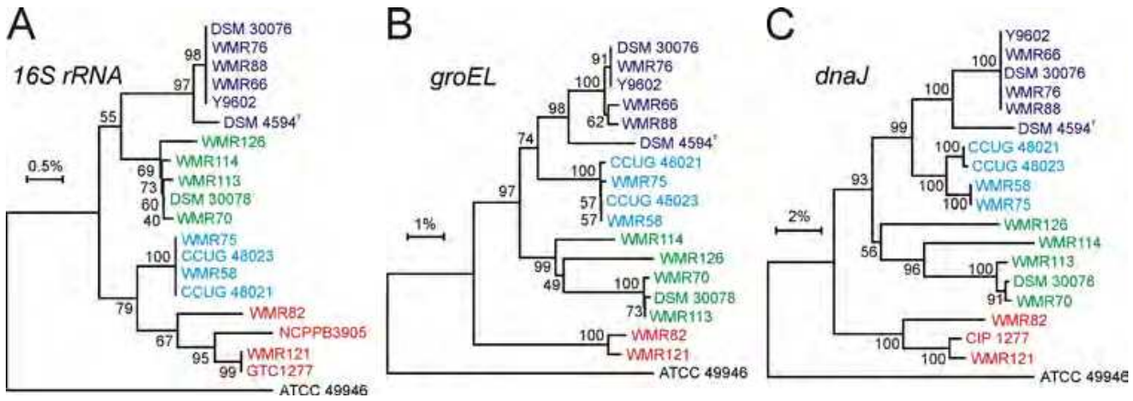

Fig. 1. Neighbour-joining trees based on partial *16S rRNA* (A), *groEL* (B) and *dnaJ* (C) gene sequences of *Rahnella* and *Ewingella*. The trees were constructed with MEGA4 (Tamura et al., 2007) using the p-distance model. Percentage bootstrap values of 1000 replicates are indicated at the corresponding nodes. The scale bars represents the indicated sequence difference. *Erwinia amylovora* ATCC 49946 was used as outgroup. Strains belonging to *Rahnella aquatilis*, *Rahnella* genomospecies 2, *Rahnella* genomospecies 3 and *Ewingella americana* are shown dark blue, light blue, green and red, respectively.

of the (partial) *16S rRNA* gene the primer pair 16S-3/16S-5 can be employed (sequences: 5´-ATATTGCACAATGGGCGC-3´ and 5´-GCCATTGTAGCACGTGTGTAG-3´, respectively; amplicon: 881 bp) (Rozhon et al., 2011). For verification a part of the *groEL* gene can be sequenced using the primer pair groEL-fwd/groEL-rev (sequences: 5´-ATGGCAGCTAAAGACGTAAAATT-3´ and 5´- TTACGACGRTCGCCRAAGC-3´, respectively; amplicon: 857 bp) (Rozhon et al., 2011). In addition a part of the *dnaJ* gene can be sequenced using the primer pair dnaJ-fwd/dnaJ-rev (sequences: 5´-CAGTATGGTCATGCAGCCTTTGAACA-3´ and 5´-TCAAAGAACTTTTTCACGCCGTC-3´, respectively; amplicon: 917 bp). Neihgbour-joining trees constructed with such sequences are shown in Figure 1. The genbank database contains numerous *Rahnella* and *Ewingella 16S rRNA* and several *groEL* and *dnaJ* gene sequences. Since little is known about the identification of most of these strains only sequences of strains deposited to strain collections should be used for analysis of the obtained data (Table 3).

Strain	Synonyms	*16S rRNA*	*groEL*	*dnaJ*
Rahnella aquatilis DSM 4594^T	CCUG 14185^T	FM876214	FM877005	HE577308
Rahnella aquatilis DSM 30076		FM876215	FM877006	HE577309
Rahnella genomospecies 2 CCUG 48021 [a]		U88434	FM877008	HE577311
Rahnella genomospecies 2 CCUG 48023		U88438	FM877009	HE577312
Rahnella genomospecies 2 CCUG 21213		FM876216	FM877007	NA
Rahnella genomospecies 3 DSM 30078 [b]	LMG 2640	U90758	FM877012	HE577310
Ewingella americana GTC 1277	DSM 4560, CCUG 14506	AB273745	NA	AB272652
Ewingella americana NCPPB 3905		X88848	NA	NA

Table 3. Accession numbers of *16S rRNA*, *groEL* and *dnaJ* gene sequences of *Rahnella* and *Ewingella* strains. Abbreviations: CCUG: Culture Collection, University of Göteborg (www.ccug.se); DSM: Deutsche Sammlung von Mikroorganismen (www.dsmz.de); GTC: Gifu Type Culture Collection; LMG: BCC/LMG Belgian Co-ordinated Collection of Microorganisms (bccm.belspo.be); NCPPB: National Collection of Plant Pathogenic Bacteria (www.ncppb.com); NA: not available. [a] Reference strain for genomospecies 2. [b] Reference strain for genomospecies 3.

2.4 Antibiotic resistance of *Rahnella* and *Ewingella*

2.4.1 Susceptibility patterns

The susceptibility patterns of more than 180 *Rahnella* strains have been described in the literature (Table 4). Many of these strains were isolated from clinical specimens but more than 75 originate from environmental samples (most of them were obtained in the study of Ruimy et al. (2010b) and in this study). *Rahnella* was found to be resistant to narrow spectrum penicillins, aminopenicillins, carboxypenicillins and most strains showed a low-level resistance to ureidopenicillins with MICs below 16 mg/l (Stock et al., 2000). Resistance was also observed for 1st and 2nd generation cephalosporins while most strains were sensitive or at least intermediate for 3rd and all strains were sensitive to 4th generation cephalosporins and carbapenems. Addition of β-lactamase inhibitors including clavulanic acid, sublactam and tazobactam decreased the MICs of all β-lactams tested. This pattern suggests the presence of a cavulainc acid-sensitive extended spectrum Ambler class A β-lactamase (Ambler, 1980) resembling the chromosomally encoded class A β-lactamase of *Klebsiella* sp. (Labia et al., 1979; Sykes & Matthew, 1976), *Escherichia hermanii* (Stock &

Antibiotic [a]	Class[b]	(Christiaens et al., 1987)	(Freney et al., 1988)	(Goubau et al., 1988)	(Harrell et al., 1989)	(Hohl et al., 1990)	(Alballaa et al., 1992)	(Hoppe et al., 1993)	(Maraki et al., 1994)	(Oh & Tay, 1995)	(Funke & Rosner, 1995)	(Matsukura et al., 1996)	(Caroff et al., 1998)	(O'Hara et al., 1998)	(Chang et al., 1999)	(Stock et al., 2000)	(Fajardo & Bueno, 2000)	(Bellais et al., 2001)	(Carinder et al., 2001)	(Tash, 2005)	(Aktaş et al., 2009)	(Ruimy et al., 2010b)	(Gaitán & Bronze, 2010)	This study
No. of strains tested		1	12	1	1	6	1	1	1	2	1	1	2	1	1	72	1	2	1	1	1	55	1	20
Amikacin	AMG						S	S	S		S					S				S	S			
Amoxicillin	APEN	R						R			R		R			R	R	R				R		R
Amoxicillin + In	APEN	S	S			SIR		S	R		S		S			S	R	S		S	S	S		S
Ampicillin	APEN	R	R	I	R		R		R	R	R	I			R				R	R	R		R	IR
Ampicillin + In	APEN															R			S	S				
Azlocillin	UPEN								S							IR								
Aztreonam	MOB								S	S								S		S	S	S	S	
Benzylpenicillin	NPEN															R								R
Carbenicillin	CPEN													S										R
Cefaclor	CEF2															SIR								IR
Cefamandole	CEF2	S							S															
Cefazolin	CEF1			R	R		R	R				S				SIR			R	S	R		S	
Cefepime	CEF4															S		S			S	S		
Cefotaxime	CEF3								S			S			S	S		I		S	S	SI		SI
Cefoxitin	CEF2		S						R				S		S	SIR		S		S	S			
Ceftazidime	CEF3								S	S			S			S		S	S	S	S	S		
Ceftriaxone	CEF3					S	S	S	S	I						SIR		S	S	S			S	
Cefoperazone	CEF3											S				SIR				S				
Cefuroxime	CEF2	S		R				R			I		I			IR		R		S				
Cephalothin	CEF1	S	R		R				R	R	R		R		R		R	R		S				IR
Chloramphenicol	O	S			S			R		S	S			S		SI					S			
Ciprofloxacin	FQU					S	S		S	S	S					S			S	S	S		S	
Fosfomycin	O							R						R		R								
Gentamycin	AMG	S			S		S	S	S	S	S	S		S	S	S			S	S	S		S	
Imipenem	CARB							S	S	S	S	S	S		S	S	S	S	S	S	S	S		
Meropenem	CARB															S		S						S
Netilmicin	AMG								S	S		S				S								
Piperacillin	UPEN								S		S		S			SIR		I	S	S	R	R	S	
Piperacillin + In	UPEN															S		S			S	S		
Tetracycline	TET			S	S				S	S	S					SIR					S			
Ticarcillin	CPEN		R							S			R			R		R		R		R	R	
Ticarcillin + In	CPEN																	S		S		S		
TMP/SMX	SUL			S	S	S	R	S	S	S		S			S	S			S	S	S		S	
Tobramycin	AMG							S	S		S					S				S			S	

Table 4. Susceptibility pattern of *Rahnella*. [a] In: β-lactamase inhibitor (clavulanic acid, sublactam or tazobactam); TMP/SMX: trimethoprim/sulfamethoxazole. [b] Classes of antibiotics: AMG: aminoglucosides; APEN: aminopenicillins; CARB: carbapenems; CEF1-4: 1st to 4th generation cephalosporins; CPEN: carboxybenicillins; FQU: flouroquinolons; MOB: monobactams; NPEN: narrow spectrum penicillins; O: other; SUL: sulfonamides; TET: tetracyclines; UPEN, ureidopenicillins. S: susceptible; I: intermediate; R: resistant.

Antibiotic [a]	Class[b]	(Pien et al., 1983)	(Bear et al., 1986)	(Pien & Bruce, 1986)	(Freney et al., 1988)	(Hohl et al., 1990)	(Heizmann & Michel, 1991)	(DeVreese et al., 1992)	(Reina et al., 1995)[c]	(Kati et al., 1999)	(Da Costa et al., 2000)[c]	(Maertens et al., 2001)	(Stock et al., 2003)	(Papaefstathiou et al., 2004)	(Ryoo et al., 2005)	(Pound et al., 2007)	(Bukhari et al., 2008)	This study
No. of strains tested		1	1	4	8	3	1	1	1	1	1	1	20	1	1	1	1	2
Amikacin	AMG	R	S	S						S			S		S	R	R	
Amoxicillin	APEN												SIR					R
Amoxicillin + In	APEN		R		SIR	IR			R	S		S	SIR	S			I	R
Ampicillin	APEN	S		S	SIR		S	S	R	S	R	S		S	S	R		R
Ampicillin + In	APEN						S				R		SI		S	R	R	
Aztreonam	MOB							S					S	S	S	R	R	
Benzylpenicillin	PEN									R	R		R					R
Carbenicillin	CPEN	S		S				S		S								R
Cefaclor	CEF2												R					R
Cefamandole	CEF2	S		S														
Cefazolin	CEF1											R	SIR			R	R	
Cefepime	CEF4									S			S		S	R	R	
Cefotaxime	CEF3	S	S	S	SI		S			S		S	S		S	I	R	S
Cefoxitin	CEF2	S	S	R	R								SIR		S	R		
Ceftazidime	CEF3		S					S		S			S			I	R	
Ceftriaxone	CEF3					S				S			S			R	R	
Cefuroxime	CEF2							S		I		I	SR			R	R	
Cephalothin	CEF1	S		R	R				R	R				R	R		R	R
Cephradine	CEF1		R															
Chloramphenicol	O	S	S	S									S	S				
Ciprofloxacin	FQU					S							S	S	S	R		
Ertapenem	CARB															R		
Fosfomycin	O												SIR					
Gentamycin	AMG	R					S			S		S				R	R	
Imipenem	CARB			S				S					S	S	S	R	R	
Levofloxacin	FQU															R		
Meropenem	CARB												S					S
Netilmicin	AMG		S					S										
Ofloxacin	FQU									S		S	S					
Piperacillin	UPEN	S		S								S	S	S	S			
Piperacillin + In	UPEN		S				S	S					S		S	I	R	
Tetracycline	TET	S	S	S							R		SI	R	S	R	R	
Ticarcillin	CPEN				R								S				R	
Ticarcillin + In	CPEN															S	R	
TMP/SMX	SUL	S		S		S		S					S	S		S	R	
Tobramycin	AMG	R	S					S				S				R	R	

Table 5. Susceptibility pattern of *E. americana*. [a, b] For codes see Table 4. [c] Only resistance information was published.

Wiedemann, 1999) and *Serratia fonticola* (Peduzzi et al., 1997). In contrast to *Rahnella*, *Escherichia hermanii* and the *Klebsiella* isolates were sensitive to 1st and 2nd generation cephalosporins while the *Serratia fonticola* β-lactamase showed activity even against 3rd generation cephalosoprins. The unique susceptibility pattern of *Rahnella* indicates an enzyme distant from the other Ambler class A β-lactamases.

Also most *Ewingella* strains are resistant to several β-lactamases, mainly 1st and 2nd generation cephalosporins, while they were sensitive to 3rd and 4th class cephalosporins. In contrast to *Rahnella* only a low or medium-level resistance for penicillins could be observed. The distribution of the MICs of these antibiotics showed a peak at the concentration range clinically defined as 'intermediate' resulting in strains that were sensitive, intermediate or resistant (Stock et al., 2003). This overlap is likely the reason that the phenotypes of ampicillin and amoxicillin resistance seem to be inconsistent in the literature (see Table 4). The β-lactamase of *Ewingella* is insensitive to inhibitors, which is typical for class C β-lactamases.

Apart from β-lactams the most remarkable resistance of *Rahnella* and *Ewingella* was for fosfomycin. The MICs of most strains exceeded 64 mg/l and often reached 512 mg/l (Stock et al., 2000; Stock et al., 2003). Also one highly resistant *Rahnella* isolate with a MIC exceeding 1600 mg/l was reported (O'Hara et al., 1998). Other resistances shared by most strains included only such to which other species of the Enterobacteriaceae are also intrinsically resistant, for instance macrolides, lincosamides and glycopeptides.

Remarkably, two multidrug resistant strains of *Ewingella* were reported. Based on an antibiogram a successful treatment with cefotetan and trimethoprim/sulfamethoxazole was initiated in one case (Pound et al., 2007), while no information about antibiotic therapy was reported in the second case (Bukhari et al., 2008). Further reports of strains with unusual susceptibility patterns are rare and usually only one or two additional resistances were observed (Table 4 and 5). Thus treatment of infections is usually simple. In several cases trimethoprim/sulfamethoxazole, ciprofloxacin, gentamycin and 3rd generation cephalosporins were successfully used. For *Rahnella* also combinations of penicillins with β-lactamase inhibitors may be an option, while this is inappropriate for *Ewingella* infections.

2.4.2 Antibiotic resistance genes and their evolution

Cloning and sequencing of the *Rahnella* β-lactamase (bla_{RAHN-1}) confirmed that it belongs to the Ambler group C (Bellais et al., 2001). The bla_{RAHN-1} gene comprises 888 bp and its translated amino acid sequence shows 75%, 71% and 67% identity to the chromosomally encoded β-lactamases of *Serratia fonticola*, *Kluyvera cryocrescens* and *Citrobacter sedlakii* and approximately 70% identity to plasmid encoded CTX-M type ESLBs found in isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumanii* and other species (Figure 2B). Currently the sequences of the complete bla_{RAHN} loci of four different strains are available. They show a similar pattern: bla_{RAHN} and its surrounding genes have the same transcriptional orientation. An upstream transcriptional regulator that may regulate bla_{RAHN} expression is lacking (Figure 2A). The expression of many chromosomally encoded class A β-lactamases including that of *Citrobacter diversus* (Jones & Bennett, 1995) and *Proteus vulgaris* (Ishiguro & Sugimoto, 1996) is regulated by LysR-type transcription factors but also some examples lacking such a control system, for instance bla_{KLUC-1} of *Kluyvera cryocrescens* (Decousser et al., 2001), are known. A recent phylogenetic study using partial β-lactamase gene sequences of *Rahnella* strains isolated from different vegetables and fruits revealed two

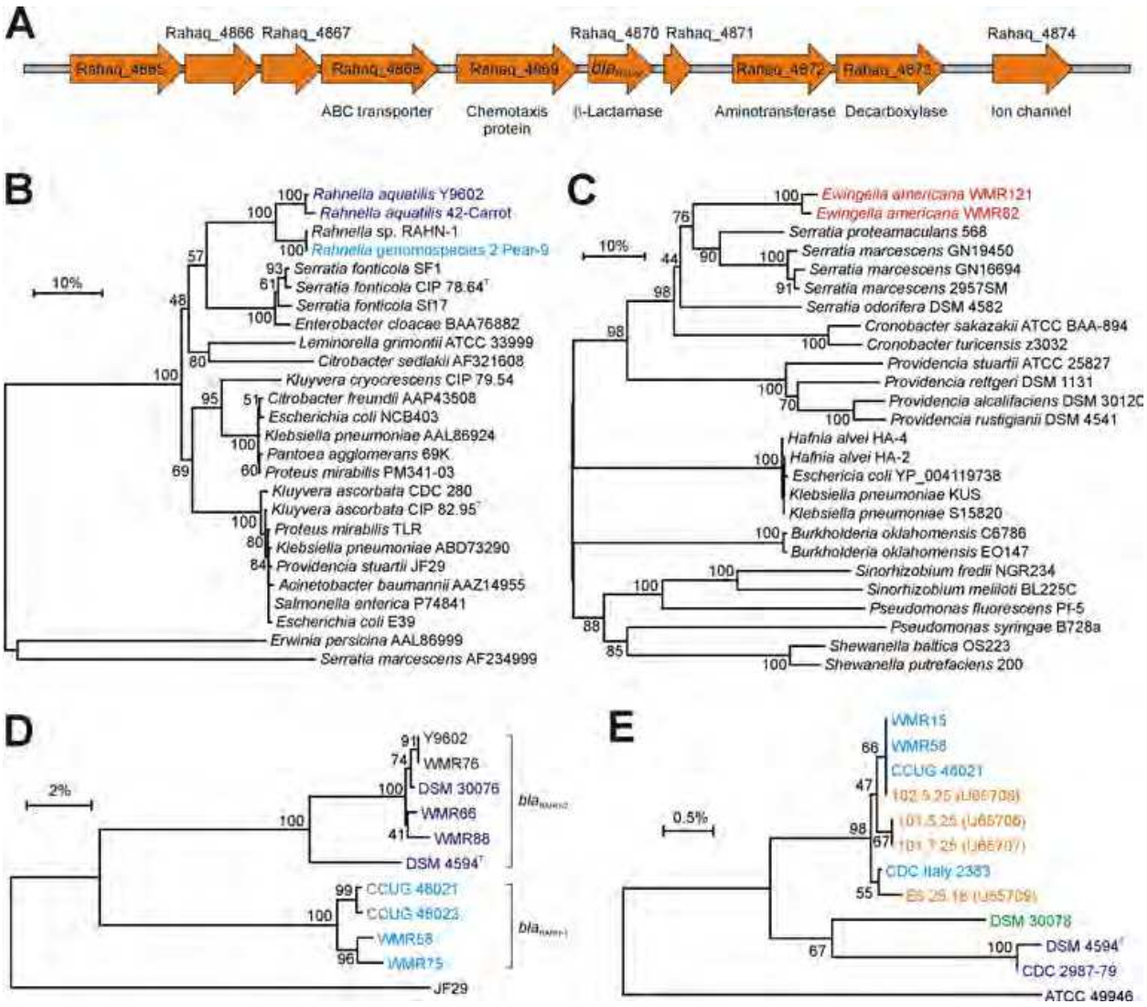

Fig. 2. The antibiotic resistance genes of *Rahnella* and *Ewingella*. (A) The bla$_{RAHN}$ locus and its surrounding genes from strain *Rahnella aquatilis* Y9602 are shown. (B) Phylogentic trees of class A β-lactamases related to *bla*$_{RHAN}$ and (C) class C enzymes related to AmpC of *Ewingella americana*. (D) β-lactamases of *Rahnella aquatilis* and *Rahnella* genomospecies 2 cluster in two different clades. *Providencia stuartii* JF29 was used as outgroup. (E) *Rahnella* isolates obtained from 12,000 year old mastodon remains (shown in orange; the accession numbers are given in brackets) cluster with recent strains belonging to *Rahnella* genomospecies 2. The tree shown is based on partial *16S rRNA* gene sequences. The same methods and colour codes like in Figure 1 were used.

clusters (Ruimy et al., 2010b). A similar dichotomy was also observed for a phlyogenetic tree based on partial *16S rRNA* and *rpoB* sequences (Ruimy et al., 2010a). The originally described *bla*$_{RHAN-1}$ gene (Bellais et al., 2001) clustered with the sequences obtained from *Rahnella* genomospecies 2. The variant found in *Rahnella aquatilis* was named *bla*$_{RHAN-2}$ (Ruimy et al., 2010b). Here we provide data confirming the results of these studies: we sequenced the (partial) *bla* gene of a number of reference strains and environmental isolates. The obtained phylogenetic tree (Figure 2D) is in agreement with that obtained for the *16S rRNA*, *groEL* or *dnaJ* gene (Figure 1). These data clearly suggest that *bla*$_{RHAN}$ was present in the ancestor before

the divergence in genomospecies. Previously the isolation of *Rahnella* strains from 12,000 year old American mastodon remains was reported. We used the partial *16S rRNA* gene sequence of these isolates and of recent reference strains to construct a phlyogenetic tree (Figure 2E). The four prehistoric strains cluster clearly with genomospecies 2. This indicates that divergence in genomospecies occured significantly more than 12,000 years ago. Thus the bla_{RHAN} seems to be present in *Rahnella* for a long time and thus represents a natural resistance of this microorganism.

However, we were unable to obtain any PCR product for strains belonging to *Rahnella* genomospecies 3 although these strains were intermediate or resistant to amoxicillin and cephalothin. Thus *Rahnella* genomospecies 3 may either possess a β-lactamase resistance gene unrelated to bla_{RAHN-1} and bla_{RAHN-2} or the primer binding sites may be different. Since the β-lactam susceptibility pattern of the three *Rahnella* genomospecies is very similar, the latter explanation seems more plausible.

Based on the susceptibility pattern an Abler class C β-lactamase was suggested for *Ewingella americana* (Stock et al., 2003). Using different primer combinations we could amplify and sequence the (partial) *ampC* gene of the strains WMR82 and WMR121. The amino acid sequence shows 72% identity to AmpC of *Serratia proteamaculans* and approximately 67% and 59% to AmpC of other *Serratia* species and to the *Providencia* cluster, respectively (Figure 2C). It is interesting to note that the AmpC sequences of the two *Ewingella* isolates share only 96.3% sequence identity. In contrast the plasmid encoded mobile β-lactamases found in some *Klebsiella pneumoniae* and *Escherichia coli* isolates exceed 98% identity (Figure 2C). It is believed that they originate from the chromosomally encoded *ampC* gene of *Hafnia alvei* (Girlich et al., 2000). This result and the observation that the vast majority of *Ewingella americana* strains have a similar susceptibility pattern suggest natural rather than acquired β-lactam resistance for this microorganism.

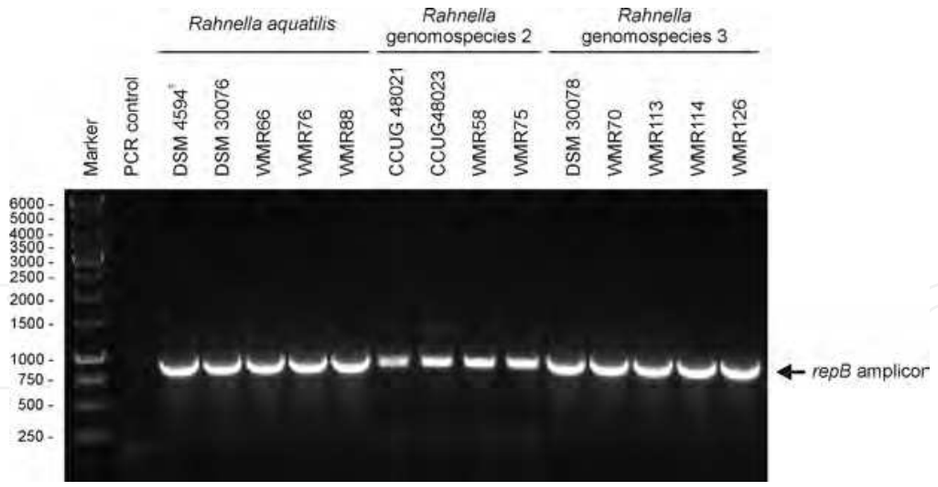

Fig. 3. The plasmid pRAHAQ01 is ubiquitously present in *Rahnella*. The (putative) replication gene *repB* of plasmid pRAHAQ01 could be detected by PCR in all strains tested.

While the molecular basis of β-lactam resistance is well known, the genotype of the fosfomycin resistance remains elusive. The high level of fosfomycin resistance observed in several strains and the report of successful transfer of the fosfomycin resistance to *Serratia marcescens* (O'Hara et al., 1998) rather suggest the presence of a specific fosfomycin:glutation-S-transferase than mutations in the GlpT, a transporter necessary for entry of fosfomycin into the cell.

2.4.3 The plasmid complement of *Rahnella*

Originally bla_{RAHN-1} was thought to be chromosomally encoded, since transfer experiments to *Escherichia coli* failed (Bellais et al., 2001). The recently completed *Rahnella* genome sequencing project showed unambiguously that the β-lactamase gene of strain Y9602 is located on a 617 kb megaplasmid, pRAHAQ01. The bla_{RAHN-2} locus and the surrounding genes of pRAHAQ01 share striking homology to three previously reported bla_{RAHN-1} and bla_{RAHN-2} sequences (Bellais et al., 2001; Ruimy et al., 2010b), indicating that they may also be plasmid born. To investigate this in more detail we analysed the sequence of pRAHAQ01 for putative plasmid replication genes and found only one candidate: Rahaq_4731 or *repB*. RepB shares 82% amino acid sequence identity with the replication protein of pEA29, a large plasmid of the plant pathogen *Erwinia amylovora* (McGhee & Jones, 2000). PCR analysis using primers for a conserved part of the *repB* gene showed a positive result for all strains tested (Figure 3). Moreover, in a previous study the presence of 400 kb to 700 kb megaplasmids in *Rahnella* soil isolates has been described (Evguenieva-Hackenberg & Selenska-Pobell, 1995). This substantiates that bla_{RAHN} may be commonly plasmid encoded. pRAHAQ01 and a second large plasmid found in strain Y9602 seem to be immobile since no known transfer system could be found on their backbones. Furthermore, no evidence could be found that bla_{RAHN} is located on a transposon or an integron.

A number of *Rahnella* strains possess also small plasmids. The majority of them were found to belong to the ColE1 family but also some ColE2 and rolling circle plasmids were isolated. Interestingly, the *Rahnella* ColE1 plasmids formed a distinct cluster in the ColE1 family and lacked any mobilisation system, suggesting that they rarely spread by horizontal gene transfer events. The ColE2 and the rolling circle plasmids possessed mobilisation systems but, like the ColE1 plasmids, were cryptic and did not encode any resistance gene (Rozhon et al., 2010).

Taken together these results suggest that the *Rahnella* β-lactamase, although plasmid encoded, is hardly mobilised to other microorganisms. Indeed, any evidence for its spread to human pathogens is currently lacking (Ruimy et al., 2010b). Similarly, also the *ampC* gene of *Ewingella* has so far remained restricted to its natural host but further experiments are necessary to rate its ability for mobilisation. Such studies would be important because previous reports provide evidence that *Ewingella americana* may be present in clinical environments (McNeil et al., 1987; Pien & Bruce, 1986) and the appearance of multiple drug resistant *Ewingella americana* strains (Bukhari et al., 2008; Pound et al., 2007) indicates that this micoorganism may exchange genetic information with human pathogens.

3. Conclusion

Rahnella is commonly associated with plants and *Ewingella* has been found at high titers in cultured mushrooms. Thus these two Enterobacteriaceae may be frequent in some types of food. Both may appear as infrequent human opportunistic pathogens. Infections are easy to treat if the specific antibiotic resistance patterns of these bacteria are considered. *Rahnella* and *Ewingella* are naturally resistant to several β-lactams, which is mediated by an Ambler class A and an Ambler class C β–lactamase, respectively. The β-lactam resistance gene of *Rahnella*, bla_{RAHN}, is located on the large non-mobile plasmid pRAHAQ01. This plasmid

belongs to the pEA29 family, which is commonly found in plant associated bacteria. *Rahnella* acquired bla_{RAHN} presumably in prehistoric times before the divergence into genomospecies. Since then bla_{RAHN} has co-evolved with its host and diverged to bla_{RAHN-1} and bla_{RAHN-2} found in *Rahnella* genomospecies 2 and in *Rahnella aquatilis*, respectively. The variant present in *Rahnella* genomospecies 3 remains to be identified. Although bla_{RAHN} is located on a plasmid it is not per se mobile and so far no hint for its mobilisation to other species has been found. However, since several examples of chromosomal resistance genes that were transferred into pathogens have been documented, it can not be excluded that also bla_{RAHN} may spread to other bacteria in the future. Based on the suceptibility pattern it was previously hypothesised that the β-lactamase of *Ewingella americana* is an Ambler class C enzyme. Here we have provided compelling data confirming this assumption. However, further studies are necessary to assess whether the *Ewingella ampC* gene is chromosome or plasmid born and its potential for transfer needs to be investigated. *Rahnella* and *Ewingella* are also naturally resistant to fosfomycin. The molecular basis of this resistance remains elusive. Other resistances were rarely reported for *Rahnella*, while recently two multidrug resistant strains of *Ewingella* were described. These characteristics should be considered for treatment of infections and for potential applications of *Rahnella* and *Ewingella*.

4. Acknowledgment

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5. References

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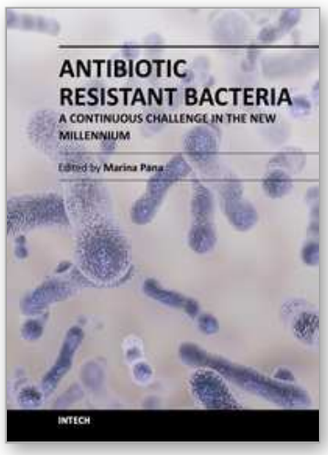

Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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