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The Natural Antibiotic Resistances of the Enterobacteriaceae \textit{Rahnella} and \textit{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 $\beta$-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 $\beta$-lactamases (EBSLs) in pathogenic Enterobacteriaceae (Canton & Coque, 2006). The CTX-M enzymes are believed to originate from \textit{Klyvera ascorbata} and \textit{Klyvera georgiana} chromosomal $\beta$-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 & Pemberdy, 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 imped ing 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, USA    | NA       | NA                 | Burn wound | Burn                 | NA        | NA      | (Farmer et al., 1985) |
| 2    | 1986, USA    | 37 y, M  | Cough, fever, right 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, lymphoma, bronchopulmonary infection with Pseudomonas | 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 | Gentamicin, azlocillin, fluclaxadil; amikacin, ceftriaxone, vancomycin | Cure | (Hoppe et al., 1993) |
| 8    | 1994, 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 | (Furke & Rosner, 1995) |
| 10   | 1995, Singapore | 48 y, M  | Fever (38.2°C) | Blood | Diabetes mellitus (for 2 yr), pulmonary tuberculosis, appendicular abscess | Ampicillin, gentamycin, amoxicillin-clavulanate, gentamycin | Cure | (Oh & Tay, 1995) |
| 11   | 1995, Singapore | 57 y, M  | Fever | Blood | Laryngeal carcinoma, total laryngectomy | Metronidazole, ceftriaxone; gentamycin | Cure | (Oh & Tay, 1995) |
| 12   | 1996, Spain  | 2 y, F   | Acute gastroenteritis | Faeces | None | None | Cure | (Reina & Lopez, 1996) |
| 13   | 1996, Spain  | 2 y, M   | Acute gastroenteritis | Faeces | AIDS | None | Cure | (Reina & Lopez, 1996) |
Table 1. Infections caused by *Rahnella*. All cases we could find in the literature are included. a Year of death (not available); b The isolates were obtained in the 1990s; c *Pantoea agglomerans* is considered as the reason.

| Case | Year, country | Age, sex | Signs and symptoms | Site | Underlying condition(s) | Treatment |
|------|---------------|----------|--------------------|------|-------------------------|-----------|
| 14   | 1996, Japan   | 11 m, F  | Fever (39.7°C), cough | Blood | Congenital heart disease | Cefpodoxime-proxetil, cefotaxime; ceftazidime, netilmicin, gentamicin; ticarcillin-clavulanate, vancomycin |
| 15   | 1997, France  | 32 y, F  | Fever (>38°C) | Blood | Ingestion of a caustic product, parenteral nutrition via a catheter | Removal of the catheter |
| 16   | 1997, France  | 61 y, M  | Fever (40°C) | Blood | Relapse from a renal carcinoma (status post), parenteral nutrition via a catheter | |
| 17   | 1998, 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, imipenem |
| 19   | 1999, Tunisia | 65 y, F  | Fever (38.5°C), ketosis | Blood | Diabetes mellitus for 5 y | Cefotaxime, trimethoprim-sulfamethoxazole, gentamycin |
| 20   | 2000, USA     | 46 y, M  | Fever | Blood | B-cell lymphoblastic leukaemia, immunosuppressive medication, Hickman catheter | Piperacillin-tazobactam, amikacin |
| 21   | 2000, Spain   | 63 y, M  | Fever (37.8°C), excessive exudate | Tracheostomy | Laryngeal carcinoma (status post) | Amoxicillin-clavulanate, cefotaxime, amikacin |
| 22   | 2003, NA      | NA, F    | NA | Urine | Co-infection with *Candida albicans* | None |
| 23   | 2004, USA     | 76 y, M  | Fever (39.8°C) | Blood | Benign prostatic hypertrophy, bacteria in urine | Tetracycline, levofloxacin |
| 24   | 2009, Turkey  | 57 y, F  | Ulcerated foot wound | Wound | Diabetes mellitus for 20 years, co-infection with *Candida* sp. | Ampicillin-subactam, augmentation of antibiotic therapy |
| 25   | 2009, Italy   | 78 y, M  | Fever, sepsis | Blood | Hospitalised at an intensive care unit, co-infection with *Candida famata* and *Pantoea agglomerans* | Meropenem |
| 26   | 2011, USA     | 27 y, F  | Septic shock, fever (38.1°C) | Blood | Sickle cell disease, central venous catheter | Ciprofloxacin, removal of the catheter |
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 | 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+, 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 | None | (Bear et al., 1986) |
| 7    | 1985, Germany | 30 y, F | adhesive eyelids, itching conjunctivae (swab) | Conjunctivae (swab) | Surgery of the gallbladder; also *Pseudomonas aeruginosa, Candida albicans* and *Serratia marcescens* were isolated from the patient | Amoxicillin-clavulanate | Cure | (Heizmann & Michel, 1991) |
| 8    | 1991, Belgium | 75 y, M | Cholecystitis, fever (39.4°C) | Blood | None | Gentamicin, trimethoprim-sulfamethoxazole | Cure | (DeVreese et al., 1992) |
| 9    | 1991+, Spain | 31 y, M | Balanitis | Penile exudate | HIV, intravenous drug abuse, several opportunistic infections | Tobramycin | Cure | (Sanmartin Jimenez et al., 1991) |
| 10   | 1995+, Spain | 18 m, M | Acute gastroenteritis | Faeces | None | None | Cure | (Reina et al., 1995) |
| 11   | 1999+, 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, amikacin |
| 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 catheter |
| 14   | 2000, Brasilia| 38 y, F  | Keratoconjunctivitis| Conjunctivae (swab) | Soft contact lens | Ciprofloxacin |
| 15   | 2003, Germany | 74 y, F  | Waterhouse-Friderichsen syndrome | Blood from heart and spleen | Pain in the left leg; otherwise healthy | Tramadol (for treatment of pain) |
| 16   | 2004, Greece  | 72 y, M  | Fever (38.5°C), diffuse abdominal pain | Peritoneal effluent | End-stage renal failure, dialysis for 3 years | Ceftazidime, tobramycin |
| 17   | 2005, 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, isepamicin |
| 19   | 2007, USA     | 77 y, F  | Shortness in breath | Sputum| Infection with *Mycobacterium tuberculosis* and *M. avium* (status post); Cohen’s disease | Trimethoprim-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 described |

Table 2. Infections caused by *Ewingella*. All cases we could find in the literature are included. a Year of birth 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 woman (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.](image-url)
of the (partial) 16S rRNA gene the primer pair 16S-3/16S-5 can be employed (sequences: 5’-ATATTGCACAATGGGCGC-3’ and 5’-GCCATTGTAGCAGGTGTAAG-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’-TTACGACCGRTGACGAAC-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’-CATATTGCTCATGCAGCCTTTGAACA-3’ and 5’-TCAAGAACITTTTCAAGCCGT-3’, respectively; amplicon: 917 bp). Neighbour-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 Accession | groEL Accession | dnaJ Accession |
|--------------------------|---------------------------|--------------------|-----------------|----------------|
| Rahnella aquatilis DSM 4594 | CCUG 14185[^a]            | FM876214           | FM877005        | HE577308       |
| Rahnella aquatilis DSM 30076 |                           | FM876215           | FM877006        | HE577309       |
| Rahnella genomospecies 2 CCG 48021[^a] |                   | U88434             | FM877008        | HE577311       |
| Rahnella genomospecies 2 CCG 48023 |                           | U88438             | FM877009        | HE577312       |
| Rahnella genomospecies 2 CCG 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                | NA              |               |
| Ewingella americana NCPPB 3905 | X88848       | NA                | NA              |               |

[^a]: Reference strain for genomospecies 2.  
[^b]: Reference strain for genomospecies 3.

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.

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 1<sup>st</sup> and 2<sup>nd</sup> generation cephalosporins while most strains were sensitive or at least intermediate for 3<sup>rd</sup> and all strains were sensitive to 4<sup>th</sup> 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 &
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| Antibiotic  | Class  | No. of strains tested | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 | 116 | 117 | 118 | 119 | 120 |
|-------------|--------|-----------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---
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Table 5. Susceptibility pattern of *E. americana*. a, b For codes see Table 4. c Only resistance information was published.

| Antibiotic                | Class       | AMG | R | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
|--------------------------|-------------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amikacin                 | AMG         | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Amoxicillin              | APEN        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Amoxicillin + In         | APEN        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ampicillin               | APEN        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Aztreonam                | MOB         | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Benzylopenicillin        | PEN         | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Carbenicillin            | CPEN        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefclor                  | CEF2        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefamandole              | CEF2        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefazolin                | CEF1        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefepine                 | CEF4        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefotaxime               | CEF3        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefotaxime               | CEF2        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ceftriaxone              | CEF3        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cefuroxime               | CEF2        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cephalothin              | CEF1        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Cephadrine               | CEF1        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Chloramphenicol          | O           | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ciprofloxacin            | FQU         | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ertapenem                | CARB        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Fosfomycin               | O           | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Gentamycin               | AMG         | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Imipenem                 | CARB        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Levofloxacin             | FQU         | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Meropenem                | CARB        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Netilmicin               | AMG         | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ofloxacine               | FQU         | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Piperacillin             | UPEN        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Piperacillin + In        | UPEN        | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Tetracycline             | TET         | S   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ticarcillin              | CPEN        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Ticarcillin + In         | CPEN        | R   | S | S | S | S | S | R | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| TMP/SMX                 | SUL         | S   | S | S | S | S | S | S | R | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |

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 1\textsuperscript{st} and 2\textsuperscript{nd} generation cephalosporins while the Serratia fonticola β-lactamase showed activity even against 3\textsuperscript{rd} generation cephalosporins. 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 1\textsuperscript{st} and 2\textsuperscript{nd} generation cephalosporins, while they were sensitive to 3\textsuperscript{rd} and 4\textsuperscript{th} class cephalosporins. 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.

Remarkingly, 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 3\textsuperscript{rd} 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\textsubscript{RAHN-1}) confirmed that it belongs to the Ambler group C (Bellais et al., 2001). The bla\textsubscript{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\textsubscript{RAHN} loci of four different strains are available. They show a similar pattern: bla\textsubscript{RAHN} and its surrounding genes have the same transcriptional orientation. An upstream transcriptional regulator that may regulate bla\textsubscript{RAHN} expression is lacking (Figure 2A). The expression of many chromosomally encoded class A β-lactamas 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\textsubscript{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*<sub>RHAN</sub> locus and its surrounding genes from strain *Rahnella aquatilis* Y9602 are shown. (B) Phylogenetic trees of class A β-lactamases related to *bla*<sub>RHAN</sub> 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 phylogenetic tree based on partial 16S rRNA and *rpoB* sequences (Ruimy et al., 2010a). The originally described *bla*<sub>RHAN-1</sub> gene (Bellais et al., 2001) clustered with the sequences obtained from *Rahnella* genomospecies 2. The variant found in *Rahnella aquatilis* was named *bla*<sub>RHAN-2</sub> (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*<sub>RHAN</sub> 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 phylogenetic 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* \text{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* \text{RAHN-1} and *bla* \text{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 *Haftia 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*<sub>RAHN</sub>-<sub>1</sub> 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*<sub>RAHN</sub>-<sub>2</sub> locus and the surrounding genes of pRAHAQ01 share striking homology to three previously reported *bla*<sub>RAHN</sub>-<sub>1</sub> and *bla*<sub>RAHN</sub>-<sub>2</sub> 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 (Evgenieva-Hackenberg & Selenska-Pobell, 1995). This substantiates that *bla*<sub>RAHN</sub> 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*<sub>RAHN</sub> 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 CoIE1 family but also some CoIE2 and rolling circle plasmids were isolated. Interestingly, the *Rahnella* CoIE1 plasmids formed a distinct cluster in the CoIE1 family and lacked any mobilisation system, suggesting that they rarely spread by horizontal gene transfer events. The CoIE2 and the rolling circle plasmids possessed mobilisation systems but, like the CoIE1 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 microorganism 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*<sub>RAHN</sub>, is located on the large non-mobile plasmid pRAHAQ01. This plasmid
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Belongs to the pEA29 family, which is commonly found in plant associated bacteria. *Rahnella* acquired blαRAHN presumably in prehistoric times before the divergence into genomospecies. Since then blαRAHN has co-evolved with its host and diverged to blαRAHN-1 and blαRAHN-2 found in *Rahnella* genomospecies 2 and in *Rahnella aquatilis*, respectively. The variant present in *Rahnella* genomospecies 3 remains to be identified. Although blαRAHN 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 blαRAHN may spread to other bacteria in the future. Based on the susceptibility 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*.

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