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Frequency and diversity of small cryptic plasmids in the genus Rahnella

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Abstract

Background: Rahnella is a widely distributed genus belonging to the Enterobacteriaceae and frequently present on vegetables. Although Rahnella has interesting agro-economical and industrial properties and several strains possess antibiotic resistances and toxin genes which might spread within microbial communities, little is known about plasmids of this genus. Thus, we isolated a number of Rahnella strains and investigated their complements of small plasmids.

Results: In total 53 strains were investigated and 11 plasmids observed. Seven belonged to the ColE1 family; one was ColE2-like and three shared homology to rolling circle plasmids. One of them belonged to the pC194/pUB110 family and two showed similarity to poorly characterised plasmid groups. The G+C content of two rolling circle plasmids deviated considerably from that of Rahnella, indicating that their usual hosts might belong to other genera. Most ColE1-like plasmids formed a subgroup within the ColE1 family that seems to be fairly specific for Rahnella. Intriguingly, the multimer resolution sites of all ColE1-like plasmids had the same orientation with respect to the origin of replication. This arrangement might be necessary to prevent inappropriate synthesis of a small regulatory RNA that regulates cell division. Although the ColE1-like plasmids did not possess any mobilisation system, they shared large parts with high sequence identity in coding and non-coding regions. In addition, highly homologous regions of plasmids isolated from Rahnella and the chromosomes of Erwinia tasmaniensis and Photorhabdus luminescens could be identified.

Conclusions: For the genus Rahnella we observed plasmid-containing isolates at a frequency of 19%, which is in the average range for Enterobacteriaceae. These plasmids belonged to different groups with members of the ColE1-family most frequently found. Regions of striking sequence homology of plasmids and bacterial chromosomes highlight the importance of plasmids for lateral gene transfer (including chromosomal sequences) to distinct genera.

Background

Rahnella, a genus of the Enterobacteriaceae, is commonly found in the rhizosphere [1,2] and phyllosphere [3], seeds [4], fruits [5,6], water [7] and intestinal tracts of herbivores including snails, slugs, and even American mastodon remains [8,9]. In addition, Rahnella strains have been isolated from the extreme environment of uranium and nitric acid contaminated soil adjacent to disposal ponds of the DOE Field Research Centre in the Oak Ridge National Laboratory Reservation in Tennessee [10]. The genus Rahnella comprises three closely related species that cannot be phenotypically differentiated: Rahnella aquatilis, Rahnella genomospecies 2 and Rahnella genomospecies 3 [8].

In recent years interest in certain Rahnella strains increased because of their remarkable properties. Rahnella might be useful for control of plant pathogens like Erwinia amylovora causing fire blight of apple trees or Xanthomonas campestris, the causal agent of black rot [11,12]. Several strains seem to improve plant nutrition, as they are able to fix nitrogen [2] and to solubilise hydroxyapatite, thus converting phosphate to a plant utilisable form [13]. The production of polysaccharides, especially levan and lactan, by different Rahnella isolates is intensively studied, because these macromolecules have ideal properties for industrial applications [14-16]. Some reports have described Rahnella as an
opportunist human pathogen but infections with *Rahnella* are usually limited to immunocompromised patients and recovery is rapid [17-19]. However, antibiotic resistances and enterotoxins encoded by several strains [20-22] might spread within microbial communities. Thus, an improved understanding of mobile genetic elements of *Rahnella* is crucial to assess the potential of lateral gene transfer to other species including human pathogens. Nevertheless, although *Rahnella* is widely distributed and frequent on vegetables and therefore likely to be routinely present in the human diet, little is known about plasmids of this genus. So far only one *Rahnella* plasmid, pHW15, has been characterised [6]. pHW15 belongs to the ColE1 family, is non-mobile and stably maintained even in the absence of selective pressure. To gain insights into the frequency, diversity and evolution of small (less than 15 kb) *Rahnella* plasmids, we isolated strains from different geographic origins and sample materials. Most plasmids belonged to the ColE1 family but we also found members of other groups, including plasmids replicating by the rolling circle mechanism. In addition, sequence analysis provided evidence for lateral gene transfer within *Rahnella* as well as between *Rahnella* and other genera.

**Results and Discussion**

**Isolation of strains, screening for plasmids and cloning**

Forty five *Rahnella* strains were isolated from vegetables obtained from supermarkets or sampled from fields. Isolates from the same sample were only included in the collection if they differed in at least one biochemical characteristic or the partial 16S rRNA gene sequence to avoid multiple sampling of the same strain. This collection was complemented by 6 strains obtained from culture collections and two strains that had been previously investigated for plasmid content (Table 1). Thus, in total 53 strains were included in this study and 10 of them (19%) contained small plasmids, as revealed by DNA isolation and subsequent gel electrophoresis. Nine of these strains contained one plasmid and one of them had two. Therefore, 10 novel plasmids were detected in addition to pHW15. Their sizes ranged from 2.9 to 7.0 kb, which is typical for small plasmids from enterobacteria [23]. The method we used for detection of plasmid DNA preferentially selects for small plasmids (below 20 - 30 kb) rather than large DNA molecules. Thus, the presence of large plasmids in the investigated strains cannot be excluded. Cloning and sequencing of the isolated plasmids revealed that the majority of them (7 of 11; 64%) belonged to the ColE1 group (plasmids pHW15 to pHW42, Fig. 1). In addition, one ColE2-like plasmid (pHW66) was isolated. The three remaining plasmids (pHW121, pHW104 and pHW126) are likely to replicate by the rolling circle mechanism. pHW121 belonged to the well-described pC194/pUB110 family, while pHW104 and pHW126 showed homology to different groups of poorly characterised plasmids.

**ColE1-like plasmids**

The replication regions of the ColE1-like plasmids showed the typical elements: RNA I, RNA II, a single strand initiation site (ssi) and a terH sequence for termination of lagging-strand synthesis. Phylogenetic analysis based on the RNA I sequence revealed that pHW15, pHW120, pHW114A, pHW114B, pHW30076 and pHW4594 represented a subgroup within the ColE1 family together with pECA1039, a plasmid isolated from *Pectobacterium atrosepticum* [24]. pHW42 did not fall into this subgroup and was more related to other ColE1-like plasmids (Fig. 2A). Not only the replication regions but also the multimer resolution sites (mrs) were closely related in all ColE1-like plasmids of *Rahnella*. In a phylogenetic tree based on mrs sites (Fig. 2B) most plasmids isolated from *Rahnella* formed a cluster similar to the RNA II tree, confirming that they form a separate class within the ColE1 family.

It might be thought surprising that all multimer resolution sites of plasmids depicted in Fig. 1 are in the same orientation with respect to the replication origin (oriV). This is also true for all ColE1-like plasmids in Fig. 2A. The explanation for this observation may lie in the intimate association of replication control and multimer resolution in the stable maintenance of ColE1-like plasmids. Because all of the ColE1 replication origins in a cell function independently, plasmid dimers (which have two origins) replicate twice as often as monomers. As a result, dimers accumulate rapidly and clonally in a process known as the dimer catastrophe [25]. RNAI-RNAII copy number control counts origins rather than plasmids, so a dimer is not differentiated from two monomers. Consequently the copy number (i.e. the number of independent molecules) of dimers is approximately half that of monomers. ColE1 lacks active partition, so plasmid stability requires the maintenance of a high copy number. As a result the copy number depression caused by dimer accumulation causes plasmid instability [26]. One part of the solution to this problem is the resolution of dimers or higher multimers to monomers by site-specific recombination. The multimer resolution site of ColE1 (designated cer, for ColE1 resolution) contains binding sites for the host-encoded recombinase XerCD and the accessory protein ArgR (Fig. 2C). They act together with PepA (whose binding site is less clearly defined) to convert dimers to monomers by site-specific recombination [27-30]. Conserved A-T tracts phased at approximately 10.5 bp intervals facilitate the curvature of the region between the ArgR and XerC/XerD binding sites, which is thought to be
| Strain          | Genomic G+C content<sup>b</sup> | Plasmid | Source                  | Year of isolation | Geographic region          | Reference |
|-----------------|----------------------------------|---------|-------------------------|-------------------|-----------------------------|-----------|
| DSM 4594<sup>c</sup> | 51.7 ± 0.5                        | pHW4594 | Water                   | Before 1976       | France                      | [60]      |
| DSM 30076       | 51.4 ± 0.4                        | pHW30076| Chicken                 | 1984 - 1988       | Not given                   | [8]       |
| DSM 30078       | -                                | -       | Minced meat             | 1984 - 1988       | Not given                   | [8]       |
| CCUG 21213<sup>3</sup> | -                                | -       | Human burn              | 1984 - 1988       | USA                         | [8]       |
| CCUG 48021<sup>3</sup> | -                                | -       | Snail, intestinal content | 1984 - 1988       | Germany                      | [8]       |
| CCUG 48023<sup>3</sup> | -                                | -       | Human blood             | 1984 - 1988       | Germany                      | [8]       |
| WMR15           | 51.9 ± 0.9<sup>3</sup>            | pHW15   | Pear, fruit             | 2000              | Austria                      | [6]       |
| WMR39           | -                                | -       | Carrot, root            | 2002              | Austria                      | This study|
| WMR41           | -                                | -       | Carrot, root            | 2002              | Austria                      | This study|
| WMR42           | 51.5 ± 0.2                        | pHW42   | Carrot, root            | 2002              | Spain                       | This study|
| WMR52           | -                                | -       | Carrot, root            | 2002              | Austria                      | This study|
| WMR58           | 51.8 ± 0.7<sup>3</sup>            | -       | Carrot, root            | 2002              | Austria                      | [6]       |
| WMR59           | -                                | -       | Leek, root              | 2002              | Austria                      | This study|
| WMR60           | -                                | -       | Leek, root              | 2002              | Austria                      | This study|
| WMR65           | -                                | -       | Spring onion, root      | 2002              | Austria                      | This study|
| WMR66           | 51.8 ± 0.6                        | pHW66   | Spring onion, root      | 2002              | Austria                      | This study|
| WMR67           | -                                | -       | Celery, root            | 2002              | Austria                      | This study|
| WMR70           | -                                | -       | Celery, root            | 2002              | Austria                      | This study|
| WMR75           | -                                | -       | Sugar beet, root        | 2002              | Austria, Lower Austria       | This study|
| WMR76           | -                                | -       | Sugar beet, root        | 2002              | Austria, Lower Austria       | This study|
| WMR77           | -                                | -       | Yellow carrot, root      | 2002              | Austria                      | This study|
| WMR79           | -                                | -       | Yellow carrot, root      | 2002              | Austria                      | This study|
| WMR81           | -                                | -       | Yellow carrot, root      | 2002              | Austria                      | This study|
| WMR82           | -                                | -       | Parsley, root           | 2002              | Austria                      | This study|
| WMR83           | -                                | -       | Parsley, root           | 2002              | Austria                      | This study|
| WMR84           | -                                | -       | Beetroot, root          | 2002              | Austria                      | This study|
| WMR86           | -                                | -       | Beetroot, root          | 2002              | Austria                      | This study|
| WMR87           | -                                | -       | Horseradish, root       | 2002              | Austria                      | This study|
| WMR88           | -                                | -       | Horseradish, root       | 2002              | Austria                      | This study|
| WMR93           | -                                | -       | Radish, root            | 2002              | Austria                      | This study|
| WMR94           | -                                | -       | Carrot, root            | 2002              | Spain, Gran Canaria          | This study|
| WMR95           | -                                | -       | Carrot, root            | 2002              | Spain, Gran Canaria          | This study|
| WMR97           | -                                | -       | Carrot, root            | 2002              | Spain, Gran Canaria          | This study|
| WMR98           | -                                | -       | Carrot, root            | 2002              | Spain, Gran Canaria          | This study|
| WMR100          | -                                | -       | Celery, root            | 2003              | Germany                      | This study|
| WMR102          | -                                | -       | Carrot, root            | 2003              | Germany                      | This study|
| WMR104          | 52.2 ± 0.3                        | pHW104  | Carrot, root            | 2003              | Germany                      | This study|
| WMR105          | -                                | -       | Carrot, root            | 2003              | Germany                      | This study|
| WMR106          | -                                | -       | Carrot, root            | 2003              | Italy                        | This study|
| WMR107          | -                                | -       | Carrot, root            | 2003              | Italy                        | This study|
| WMR108          | -                                | -       | Carrot, root            | 2003              | Italy                        | This study|
| WMR109          | -                                | -       | Potato, tuber           | 2003              | Egypt                        | This study|
| WMR113          | -                                | -       | Leek, root              | 2003              | Belgium                      | This study|
| WMR114          | 51.3 ± 0.2                        | pHW114A+B| Leek, root             | 2003              | Belgium                      | This study|
| WMR120          | 52.6 ± 0.5                        | pHW120  | Carrot, root            | 2005              | Spain, Tenerife              | This study|
| WMR121          | 52.5 ± 0.5                        | pHW121  | Carrot, root            | 2005              | Spain, Tenerife              | This study|
| WMR126          | 52.2 ± 0.1                        | pHW126  | Carrot, root            | 2006              | Albania                      | This study|
| WMR128          | -                                | -       | Carrot, root            | 2006              | Croatia, Dubrovnik           | This study|
| WMR138          | -                                | -       | Carrot, root            | 2006              | Spain, La Palma              | This study|
beneficial for recombination complex formation [31,32]. These sequence elements are conserved in the mrs sites of the CoIE1-like plasmids (Fig. 2C).

Multimer resolution is necessary but not sufficient to combat the threat of the dimer catastrophe. A checkpoint, mediated by the small regulatory transcript Rcd, ensures that the cell does not divide before multimers have been resolved completely to monomers [33]. Rcd binds to the enzyme tryptophanase, stimulating the production of indole which inhibits cell division by an unknown mechanism [34]. Rcd is expressed from the 
Pcer promoter within cer. 
Pcer is active in plasmid multimers but is repressed in monomers by FIS and XerCD [35]. A FIS binding site important for regulation of 
Pcer has been mapped recently [35] (Fig. 2C).

As stated above, we observed that the multimer resolution sites of the CoIE1-like plasmids were in the same orientation with respect to the replication origin (Fig. 1). In other words RNAII and rcd are invariably transcribed in the same direction. A possible explanation could lie in the complex regulation of 
Pcer. FIS is required for high fidelity repression of the promoter in plasmid monomers but it is the lifting of XerCD-mediated repression in plasmid dimers which is thought to induce synthesis of Rcd and the inhibition of cell division [35].

The main evidence supporting this hypothesis is that, while the mutational inactivation of either XerC or XerD in a cell containing plasmid monomers gave a substantial increase in Rcd expression, there was no induction of Rcd expression when ArgR or PepA was inactivated [35]. RNAII read-through transcription entering cer (or the mrs on related plasmids) would first displace ArgR/PepA which will ensure that 
Pcer remains inactive. If, however, cer was in the opposite orientation, transcription might displace XerCD, inducing transient expression of Rcd from plasmid monomers. A similar argument can be made for the progress of the replication fork through cer. In the existing orientation the fork will displace ArgR before XerCD, thus ensuring that 
Pcer remains repressed during replication. Moreover, active 
Pcer facing in the opposite direction might transiently stall the replication fork, since active promoters can act as replication barriers [36,37].

In addition to the replication unit and the mrs all sequenced CoIE1-like plasmids possessed a conserved open reading frame with homology to excI of CoIE1 (Fig. 1 and Additional file 1). ExcI was originally believed to mediate entry exclusion of homologous plasmids [38] but later it was convincingly shown that mbeD exhibits this activity [39]. Therefore the function of ExcI remains unknown.

In addition to these general features most CoIE1-like plasmids contained highly conserved regions as indicated in Fig. 1. Clearly these plasmids show a highly mosaic structure. Since pHW114A and pHW114B reside in the same strain, their similarity can be potentially explained by recent recombination events in their host. However, the structures of the other plasmids argue strongly for frequent horizontal transfer within Rahnella and between Rahnella and Pectobacterium, the host of pEC1A1039. Interestingly, none of the CoIE1-like plasmids from Rahnella possessed any known mobilisation system.

pHW66 is a CoIE2-like plasmid
pHW66, like the CoIE1-family plasmids, showed a hybrid structure. It possessed a CoIE2-like replication system composed of a repA gene encoding the replication protein and a conserved nucleotide sequence that might function as oriV (Fig. 3). While the replication origins of CoIE2-like plasmids are usually located immediately downstream of the repA gene [40], the putative oriV of pHW66 was separated from repA by an insertion of more than 2 kb with an unusually low G+C content of 36% (the host strain of pHW66 has a G+C content of 51.8 ± 0.6%; Table 1). Interestingly, this insert comprised two genes that seemed to be cistronic with repA. ORF2 showed distant similarity to a putative ATPase from Shewanella woodyi and ORF3 was weakly homologous to a hypothetical protein from Lyngbya sp. (Additional file 1). Whether these genes have a role in plasmid replication or maintenance cannot be predicted.

Table 1: Strains used in this study (Continued)

| Strain   | Source                  | Year | Country       | Study             |
|----------|-------------------------|------|---------------|-------------------|
| WMR140   | -                       | 2006 | Spain, La Palma | This study       |
| WMR141   | Carrot, root            | 2007 | Portugal, Madeira | This study       |
| WMR143   | Carrot, root            | 2007 | Portugal, Madeira | This study       |
| WMR144   | Carrot, root            | 2007 | Portugal, Madeira | This study       |

a DSM strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. CCUG strains were obtained from the Culture Collection, University Göteborg, Sweden.

b Means and standard deviations of the mol% G+C contents were calculated from at least three independent measurements.
c Synonyms: CCUG 14185T, ATCC 33071T, CUETM 77-115T, MCCM 01700T, CDC 1327-79T.
d Synonyms: CDC 658-79, MCCM 01948.
e Synonyms: SM S7/1-576, CDC 4402-96.
f Synonyms: SM Bonn 7, CDC 4418-96.
g Taken from [6].

http://www.biomedcentral.com/1471-2180/10/56
An insert of low G+C content adjacent to repA has also been described for the ColE2-like plasmid pUB6060 [41] but the inserts of pHW66 and pUB6060 are distinct. Another module found on pHW66 was a mobilisation system of the ColE1-superfamily composed of a conserved transfer origin (oriT) and 4 genes: mobA, mobB, mobC and mobD [42]. Close homologues of these genes were present on pUB6060, highlighting the close relationship between pHW66 and pUB6060. It is also interesting to note that neither pHW66 nor pUB6060 possessed a XerCD-type multimer resolution system, although this type is frequent among ColE2-like plasmids [40]. The last module was located downstream of the mobilisation system and consisted of two open reading frames with remarkable homology to two consecutive genes of unknown function in the chromosome of Erwinia tasmaniensis Et1/99 [43]. The significance of this will be discussed below.

**Plasmids sharing homology to rolling circle replicons**

While the plasmids described above exhibited clear homology to previously classified plasmids, database searches with pHW121 retrieved only distantly-related sequences. The translated amino acid sequence of the largest ORF of pHW121 was 19%, 17% and 16% identical to replication proteins of pZMO1, pCA2.4 and
pUB110, respectively (Additional file 1). Importantly, the metal binding domain showed the typical signature HUHxLUxV and the catalytic domain contained the conserved Tyr residue involved in the nucleophilic attack on the plasmid DNA at initiation of replication [44], identifying orf1 as repA and pHW121 as a member of the pC194/pUB110 family. A sequence was present upstream of repA that might function as oriV (Fig. 4A).

Interestingly, the putative oriV was preceded by 16 perfect and 1 imperfect direct repeats of the sequence GGGTTTT. Such a motif has not been described so far for any pC194/pUB110-like plasmid. In addition, pHW121 possessed a putative mobilisation protein of the MOBQ family. Although the homology was low, the typical motifs were present [42]. Due to a lack of conservation no putative oriT could be identified. ORF3 of pHW121 was similar to ImcC of Legionella pneumophila. Several genes of the imc/dot complex are essential for the ability of L. pneumophila to survive in macrophages during lung infection such as Legionnaires’ disease. However, no function has so far been attributed to ImcC [45].

pHW104 showed similarity to members of a poorly studied plasmid family (Fig. 4B). A 298 amino acid protein of pHW104 showed more than 70% identity to the putative replication protein of pVCG1.2 and 22.5% identity to RepA from pAM10.6. The involvement of the latter in replication has been proven experimentally [46]. In addition pHW104 comprised a ColE1-type

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**Figure 2** The ColE1-like plasmids of *Rahnella* form a sub-family
Phylogenetic trees were constructed based on RNA II (A) or the mrs (B). In both trees most ColE1-like plasmids isolated from *Rahnella* (shown in bold letters) formed a cluster with pECA1039, a plasmid of *Pectobacterium araboplasticum*. (C) Alignment of the multimer resolution sites. The ArgR, FIS, XerC and XerD binding sites are boxed and conserved A-T stretches responsible for DNA bending are underlined. The -10 and -35 boxes of the ColE1 Pcer promoter are underlined and the start of the Rcd coding region is indicated by an arrow. Nucleotides conserved in at least 50% of the sequences are shown in bold and invariant sites are marked with an asterisk.

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**Figure 3** ColE2 origins of replication
The thick arrow indicates the primer RNA and direction of replication in ColE2-P9. Further codes as in Fig. 2.
mobilisation system (Fig. 5A) and two open reading frames of unknown function.

pHW126, the smallest plasmid found in the genus Rahnella, belonged to a novel, yet uncharacterised class of plasmids. It consisted of only 2886 bp and possessed two ORFs. ORF1 showed similarity to relaxases of the pMV158-superfamily mediating plasmid mobilisation. The characteristic motif HxDExxPHxH, as well as an invariant Arg residue in the N-terminus, were present and a putative oriT could be identified approximately 100 bp upstream of orf1 (Fig. 5B). Thus orf1 was named mob. BLAST and FASTA searches with the translated amino acid sequence of ORF2 identified homologous sequences from two uncharacterised plasmids from Klebsiella pneumoniae, pI GRK and pIGMS31, and one plasmid from Ruminobacter amylophilus, pRAO1 (55.8%, 26.1%, and 22.7% identity, respectively). Iterated PSI-BLAST searches with ORF2 from pHW126 as well as with Rep from pHW104 retrieved sequences of replication proteins from pSN2-like plasmids and pJW1, indicating that all these plasmids might form a super-family (Fig. 4B). However, the Rep sequence identity between members of different clades shown in Fig. 4B was around 10% in pair wise
Figure 5 Alignments of transfer origin (oriT) nic sites of the ColE1-superfamily (A) and the pMV158-superfamily (B)

Experimentally determined nic-cleavage sites are indicated by vertical arrows. Inverted repeats involved in formation of a stem-loop-stem structure are underlined. Other codes as in Fig. 2.
reading frames of unknown function and a repA-like gene. Interestingly, while RepA proteins encoded by ColE2-like plasmids showed a high degree of similarity from the N- to the C-terminus, the RepA-like protein of *E. tasmaniensis* Et1/99 was highly similar at the N-terminus but the last 45 amino acids were unrelated (Additional file 3). This RepA version might therefore not be functional. A BLAST search with the *E. tasmaniensis* Et1/99 region homologous to pHW66 indicated a hybrid structure: the 3’ part harbouring the two ORFs was similar to other enterobacterial chromosomes, while the 5’ part containing the truncated repA retrieved only plasmid sequences. With the full-length sequence there was no hit apart from pHW66. This region of the *E. tasmaniensis* Et1/99 chromosome might therefore be the result of a recent insertion of a part of a plasmid related to pHW66.

pHW4594 possessed a cluster of three genes, orf4, orf5 and orf6, that showed homology to an operon of the *P. luminescens* chromosome (Fig. 1). Although similar genes were also present in other genera, this particular arrangement could only be observed in *P. luminescens*. However, since the chromosome of *Rahnella* is not sequenced, this gene cluster of pHW4594 might also originate from the genome of its host. To test this hypothesis we investigated genomic DNA of several *Rahnella* strains by Southern blot analysis using a probe containing the main parts of orf5 and orf6 (Fig. 6). Only in the host strain of pHW4594, DSM 4594\(^1\), a signal could be detected which corresponded to the expected restriction fragment of the plasmid itself. Signals indicative of genomic copies of orf5 and orf6 could neither be detected in DSM 4594\(^1\), nor in any other strains of *Rahnella aquatilis*. Different strains of *Rahnella* genomospecies 1 and genomospecies 2 did not show any signal either. Thus, it is most likely that the orf4 orf5 orf6 gene cluster originates from *P. luminescens* (or another species) but not from *Rahnella*.

*Photorhabdus* is an enterobacterial symbiont of soil nematodes that infect various insects. After the nematode attacks an insect *P. luminescens* is released and produces a wide range of virulence factors ensuring rapid insect killing [52]. Recently it has been shown that *Rahnella* is the predominant species in the intestinal tract of the ghost moth *Hepialus gonggaensis* [53], indicating that *Rahnella* might frequently be present in insects. On the other hand, *E. tasmaniensis* is common on apple and pear barks and blossoms, and *Rahnella* has been isolated from apple and pear fruits [5,6,54]. Therefore, *Rahnella* seems to have overlapping habitats with *P. luminescens* and *E. tasmaniensis*, which might favour exchange of mobile genetic elements between *Rahnella* and these species.

**Conclusions**

The frequency of small (less than 15 kb) plasmids is highly variable within the Enterobacteriaceae. For instance, they are extremely rare in *Citrobacter freundii* while 42% of *Escherichia coli* isolates possess at least one plasmid [23]. For the genus *Rahnella* we observed plasmid-containing isolates at a frequency of 19%, which is in the average range. ColE1-like plasmids were the predominant family, which is typical for enterobacterial genera. Most ColE1-like plasmids from *Rahnella* formed a subgroup within the ColE1 family on the basis of RNA II or mrs-based phylogenetic trees. The mrs sites of the ColE1-plasmids were arranged in a constant orientation with respect to the replication origin. Such conservation is likely to prevent inappropriate activation of the P\(_{cer}\) promoter by read-through transcription or during replication. High-fidelity control of P\(_{cer}\) is essential because the promoter directs expression of a small RNA that causes inhibition of cell division by stimulating indole production. The ColE1-plasmids shared extensive regions of high sequence homology in coding and in non-coding regions, indicating frequent horizontal gene transfer and recombination events among plasmids within the genus *Rahnella*. Interestingly, none of the ColE1-like plasmids found in this study possessed a mobilisation system. In contrast, the other plasmids
analysed (one CoIE2-like plasmid and three rolling circle plasmids) contained mobilisation genes. pHW121 is a member of the pC194/pUB110 family. pHW104 and pHW126 belong to different groups of poorly-characterised plasmids and might form a super-family with pSN2-like plasmids and pFW1. To our surprise the plasmids lacked genes which confer an obvious benefit upon their hosts. Of course some of the genes with unknown function might encode proteins with advantageous functions but at least for some of the plasmids the term “selfish DNA” seems appropriate. The best example is pHW126, the smallest plasmid found in Rahnella. This plasmid possessed only two ORFs, a putative replication gene and one for mobilisation. Since these coding sequences cover more than 70% of the plasmid, and additional regions are expected to function as oriV and oriT, the plasmid is simply too small to bear any gene beneficial to the host. The low G+C content of this plasmid might indicate that Rahnella is not its normal host. In contrast, the close similarities among the CoIE1-like plasmids provided compelling evidence that Rahnella is their normal host. The presence of genes probably derived from P. luminescens on pHW4594 and stretches of the chromosome of E. tasmaniensis highly homologous to parts of pHW66 highlight the importance of plasmids for genetic exchange of even chromosomal sequences among different genera.

Methods

Media and growth conditions

E. coli and Rahnella strains were grown in MLB medium (10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7) at 37°C and 30°C, respectively, if not otherwise indicated. When necessary, ampicillin was added to a concentration of 100 μg/ml.

Isolation and identification of Rahnella strains

Different types of plant materials (Table 1) were homogenised in sterile PBS and dilutions plated on Levine-EMB agar (Merck, Darmstadt, Germany). After incubation at 36°C for 48 ± 8 h dark colonies were sampled and restreaked twice on MLB plates to obtain pure cultures. Strains were classified by routine biochemical tests and partial 16S rRNA gene sequencing [6]. For amplification of the 16S rRNA gene the primer pair fD2 and rP1 was employed. The PCR product was purified with a Nucleospin Kit (Macherey-Nagel, Düren, Germany) and directly sequenced using the primers 16S-3 and 16S-5. Primer sequences are shown in Additional file 4.

Cloning and sequencing of the plasmids

Presence of plasmids was investigated by standard alkaline lysis miniprep [55] and subsequent agarose gel electrophoresis. The strains WMR15 and WMR58 (Table 1) were used as positive and negative controls, respectively. Plasmid DNA for cloning was isolated with a Genomed plasmid midi kit and further purified by agarose gel electrophoresis. Plasmid DNA was digested with appropriate restriction enzymes and cloned into pBluescriptIIKS+ (Stratagene, La Jolla, CA) cut with the same enzyme or an enzyme forming compatible ends. Both strands were sequenced by primer walking. A complete sequence for each plasmid was obtained by assembling individual reads with ContigExpress from the VectorNTI package (Invitrogen, Carlsbad, CA).

Sequence annotation and phylogenetic analysis

Plasmid DNA sequences and predicted open reading frames were used for BLAST, PSI-BALST and FASTA databank searches at the genebank http://www.ncbi.nlm.nih.gov and ddbj http://www.ddbj.ac.jp websites. AlignX from the VectorNTI package was used to identify further less conserved or short elements e.g. oriV, oriT or ssi sites. The same program was employed to calculate the global identity of plasmid ORFs and sequences retrieved from databases. Phylogenetic analyses were performed with MEGA4 [56]. Neighbour-joining (NJ) trees were constructed using the p-distance model for DNA and the JTT matrix for amino acid sequences. Positions with gaps were usually completely deleted except for alignments containing highly diverse sequences, where pairwise deletion was chosen. Bootstrap values were calculated from 1000 replicates and indicated at the corresponding nodes. Almost identical tree topologies were obtained with other methods (minimum evolution and UPGMA) and models (Poisson correction, PAM). G+C contents of plasmids were calculated using ARTEMIS 10 [57].

Detection of ori deletion

pHW126 was digested with BglII and HindIII and the 1463 bp fragment containing the putative rep gene and the upstream intergenic sequences cloned into pBKanT [6] linearised with BamHI/HindIII. The resulting construct, designated pB126ΔBH, was digested with SpeI, the 446 bp fragment isolated and cloned into the same construct digested with XbaI which led to construct pB126-2ori. This construct was used to assay replication origin deletion: pB126-2ori was digested with Sall and the fragment containing the KanR gene and the pHW126 sequences isolated by agarose gel electrophoresis. The purified DNA was diluted to a concentration of 1 ng/μl and self-circularised by incubation with 1 U T4 ligase for 1 h at room temperature in a total reaction volume of 20 μl leading to pHW126-2ori. After transformation into E. coli INVα F- the cells were plated on MLB-kanamycin (30 μg/ml) plates.
and incubated overnight at 37°C. Three individual colonies were transferred completely to 100 ml MLB-Kan medium each and grown overnight. Plasmid DNA was isolated from these cultures using a Genomed plasmid midi kit as recommended by the manufacturer. Formation of plasmids containing only one pHW126 origin of replication was observed by agarose gel electrophoresis after digestion with HindIII or SalI. For confirmation, both bands were cut out, extracted with a Macherey-Nagel gel extraction kit and used as a template for PCR amplification with the primer pair pHW126-11/Kan rev. The amplification product was cleaned and directly sequenced employing the same primers as used for PCR. As a control pHW15-2ori, which possesses two pHW15 origins of replication in tandem repeat, was tested in the same way. pB15In(NsiI) was constructed by inserting pHW15 [6] linearised with NsiI into pBKanT. Subsequently, this construct was linearised with HindIII and PstI and ligated with the 1218 bp fragment obtained by digesting pBKanT-pHW15Δ(ORF1+2+3) [6] with HindIII and NsiI. This led to construct pB15-2ori which was finally digested with SalI and self-circularised to obtain pHW15-2ori.

**Southern blot analysis**

Approximately 3 μg genomic DNA were digested with an appropriate restriction enzyme and separated by agarose gel electrophoresis. After denaturation with 0.5 M NaOH, neutralisation with 5× TBE and equilibration with 1× TBE the DNA was transferred to a Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK) by semi-dry electroblotting using 1× TBE as transfer buffer. Cross linking was achieved by irradiation with 120 mJ/cm² UV of 254 nm. Subsequently, the membrane was pre-hybridised with Church buffer [58] containing 100 μg/ml freshly denatured herring sperm DNA. The probe was prepared by PCR: a 50 μl reaction contained 1 U GoTaq (Promega, Madison, WI), 10 μl 5× buffer containing Mg²⁺, 1 ng pHW4594 as template, 1 μl primer mix (pHW4594-fwd/pHW4595-rev; each 5 μM), 1 μl nucleotide mix (0.5 mM each of dATP, dGTP and dTTP and 0.05 mM dCTP) and 30 μCi [α-³²P]-dCTP (3000 Ci/mmol; PerkinElmer, Waltham, MA). After an initial denaturation step at 94°C for 5 min 35 cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 2 min were performed prior a final extension step at 72°C for 10 min. The denatured amplicon (95°C, 10 min) was added to the blocked membrane and hybridised for 18 h at 60°C. The membrane was washed 5 times with 0.05% SDS in 1× SSC [51] at 60°C and once with distilled water at room temperature. Signals were detected by autoradiography.

**Determination of genomic G+C contents**

The genomic DNA G+C contents of selected strains were determined by HPLC analysis as described previously [6].

**Nucleotide sequence accession numbers**

Plasmids sequences obtained in this study were deposited in the EMBL nucleotide sequence database with the following accession numbers: [EMBL:FN429021], pHW42; [EMBL:FN429022], pHW114A; [EMBL: FN429023], pHW114B; [EMBL:FN429024], pHW120; [EMBL:FN429025], pHW4594; [EMBL:FN429026], pHW30076; [EMBL:FN429027], pHW66; [EMBL: FN429028], pHW121; [EMBL:FN429029], pHW104; [EMBL:FN429030], pHW126. Accession numbers retrieved from databases are listed in Additional file 5.
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Authors’ contributions
WR conceived the study and was involved in all stages of experimental work and data analysis and drafted the manuscript. EP participated in strain isolation and manuscript preparation. MK participated in database searches and sequence annotation. DKS interpreted the results regarding the multimer resolution sites. BP participated in data analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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