Microevolutionary Events Involving Narrow Host Plasmids Influences Local Fixation of Vancomycin-Resistance in Enterococcus Populations

Ana R. Freitas1,2, Carla Novais1, Ana P. Tedim2,3,4, Maria Victoria Francia5, Fernando Baquero2,3,4, Luísa Peixe1, Teresa M. Coque2,3,4*

1 REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal, 2 Instituto Ramón y Cajal de Investigación Sanitaria (IRYCS), Madrid, Spain, 3 CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain, 4 Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, 5 Servicio de Microbiología, Hospital Universitario Marqués de Valdecilla e Instituto de Formación e Investigación Marqués de Valdecilla (IFIMAV), Santander, Spain

Abstract

Vancomycin-resistance in enterococci (VRE) is associated with isolates within ST18, ST17, ST78 Enterococcus faecalis (Efm) and ST6 Enterococcus faecalis (Efs) human adapted lineages. Despite of its global spread, vancomycin resistance rates in enterococcal populations greatly vary temporally and geographically. Portugal is one of the European countries where Tn1546 (vanA) is consistently found in a variety of environments. A comprehensive multi-hierarchical analysis of VRE isolates (75 Efm and 29 Efs) from Portuguese hospitals and aquatic surroundings (1996–2008) was performed to clarify the local dynamics of VRE. Clonal relatedness was established by PFGE and MLST while plasmid characterization comprised the analysis of known relaxases, rep initiator proteins and toxin-antitoxin systems (TA) by PCR-based typing schemes, RFLP comparison, hybridization and sequencing. Tn1546 variants were characterized by PCR overlapping/sequencing. Intra- and inter-hospital dissemination of Efm ST18, ST132 and ST280 and Efs ST6 clones, carrying rolling-circle (pEFNP1/pRf1) and theta-replicating (pCIIZ-like, IncI8, pHT1-like, two pRUM-variants, plG1-like, and pheromone-responsive) plasmids was documented. Tn1546 variants, mostly containing IS101 or IS1216, were located on plasmids (30–150 kb) with a high degree of mosaicism and heterogeneous RFLP patterns that seem to have resulted from the interplay between broad host IncI8 plasmids (pIP501, pRE25, pEF1), and narrow host RepA_N plasmids (pRUM, pAD1-like). TAs of IncI8 (ω-c-z) and pRUM (Axet-xe) plasmids were infrequently detected. Some plasmid chimeras were persistently recovered over years from different clonal lineages. This work represents the first multi-hierarchical analysis of VRE, revealing a frequent recombinatorial diversification of a limited number of interacting clonal backgrounds, plasmids and transposons at local scale. These interactions provide a continuous process of parapatric clonalization driving a full exploration of the local adaptive landscape, which might assure long-term maintenance of resistant clones and eventually fixation of Tn1546 in particular geographic areas.

Introduction

Since its first description in the late 80’s, vancomycin-resistant enterococci (VRE) have been increasingly reported worldwide, but presenting remarkable geographical and temporal differences in local rates [http://www.cdcdep.org/ResistancedMap/bug-drug/EFaq-VC] [1-3]. Vancomycin-resistant Enterococcus faecium (VREfm) became endemic in most North American hospitals since the mid 90’s [1,2,4-6] while their overall occurrence in Europe remained low until recently, when VRE nosocomial outbreaks started to be increasingly reported in some European countries (Annual Report of the European Antimicrobial Resistance Surveillance Network, EARS-Net, 2009) [1,5,7,8]. Despite E. faecium (Efm) being less frequently found than Enterococcus faecalis (Efs) in clinical isolates, it is far more frequently resistant to vancomycin, one of the last-line intravenous antibiotic resources for therapy. However, although the rate of vancomycin-resistant E. faecalis (VREfs) has remained low, they are steadily increasing in both the US and in EU countries [http://www.cdcdep.org/ResistancedMap/bug-drug/EFaq-VC] [3].

Vancomycin resistance among enterococci is mostly due to the spread of Tn1546 (vanA genotype) and Tn1549 (vanB genotype), which are generally identified on plasmids and chromosome, respectively [3]. The few studies in which plasmids carrying Tn1546 from human or animal isolates were characterized...
revealed they belong to plasmid families RepA_N (pheromone-responsive plasmids and derivatives of pRUM and pLG1), Inc18 and pHTB [9–18] suggesting an apparent plasmid promiscuity of this transposon influencing its dissemination among enterococcal populations.

Recent analysis of enterococcal populations in the clinical setting depicts a rugged epidemiological profile, with successive waves of isolates causing infections, which belong to specific lineages of *E. faecium* (ST17, ST18 and ST78, previously considered within the same clonal complex (CC) 17), and *E. faecalis* (ST6, ST40) [19–21]. However, regional differences in the rates of VRE cannot be only explained by clonal replacement dynamics as suggested for other pathogens [22–24].

The aim of this study was to address the dynamics of vancomycin resistance among enterococci in Portugal, one of the developed countries with higher rates of both VREfm (21–23%) and VREfs (1.8–4.1%) (www.cdc.de/p/ResisanceMap?bug-drug/Efe-VC), and where VanA is prevalent over VanB [3,25–27] by analyzing the clonal and plasmid backgrounds influencing the spread and persistence of Tn1546. Our study suggests that clonalization, the local selection of distinct clonal variants giving rise to durable bacterial lineages, might result and be modified by the local spread and recombinatorial dynamics of mobile genetic elements, thus providing new clues about the local multi-hierarchical evolutionary biology of vancomycin resistance.

**Results**

Local dynamic landscape drives the spread and fixation of vancomycin resistance in Portuguese hospitals

We have determined that the enterococcal population from the Portuguese hospitals is formed by an ensemble of MLST/PFGE clones. Efm isolates fit in three out of six phylogenomic groups recently established by using Bayesian Analysis of Population Structure (BAPS), namely BAPS groups 2, 3 and 5 [19] (Figure 1). Most of the isolates cluster into the predominant BAPS group 3 [subgroup 3–3 comprising main human lineages ST18 (ST18 and ST132) and ST17 (ST16); and subgroup 3–1 comprising ST280], and the BAPS group 2 (including ST30 and ST656/ST78 lineage, ST5/CC5, ST190/CC9), which have been previously associated with isolates from humans and both animals and humans, respectively [10,19,25–30]. A number of clones cluster in the small Efm BAPS group 5 (ST366, ST367, ST369), which seems to comprise mosaic genomes [19]. Isolates of Efs belong to ST6/CC2, ST30, ST35, ST117, and ST159 lineages although, to the date of this publication, Efs population has not been clustered in different BAPS groups. Among all them, isolates within ST18 Efm and ST6 Efs lineages were predominant, in line with the intra- and interhospital spread of particular highly transmissible Efm and Efs clones recovered in Portuguese hospitals since the late 90s [22,25,31,32]. While ST6 Efs was widely disseminated in all hospitals analyzed in this country [26], specific Efm lineages were overrepresented in Coimbra (ST18) and Oporto (ST132, a single locus variant, SLV, of ST18). Strains belonging to ST18 (showing PFGE types H70 and H78), ST132 (PFGE type H88) and ST280 (with PFGE types 71 and H100) were spread in different hospitals (Figure 1 and Figure 2).

It is worthwhile to note the possible relatedness between isolates of different STs (Figure 1 and 2). They include some isolates linked to BAPS 3–3 subgroup as ST18, ST30, ST125, ST132, ST368, ST369, all SLVs of each other, with PFGE patterns differing in less than 8 bands difference. Similarly, strains identified as ST280 and ST391, both linked to BAPS group 3–1, showed related PFGE patterns despite being trilocus variants (≤ 8 bands difference).

**vnaA-Tn1546 is located on highly transferable mosaic plasmids involving narrow host pRUM and pAD1 derivatives**

The plasmid content of the isolates studied appears in Figure 2. Efm isolates carried a variable number of plasmids (n = 1–6) which contained specific sequences of different families including rolling-circle plasmids (RCR) related to pR11 and small theta plasmids related to pCIZ2, RepA_N (pRUM-like, pLG1), pHTB (present in all ST132 isolates), and Inc18 (pRE25 and pEF1-related). All Efs contained RCR plasmids and pheromone-responsive-plasmids.

<vnaA-Tn1546 was located on plasmids ranging from 30 to 150 kb, successfully transferred by conjugation in 95% (n = 71/75) of Efm and 97% (n = 28/29) of Efs, with a variable frequency (10^{−1}–10^{−6}). Transferable plasmids were identified as members of pRUM and Inc18 families or were mosaic plasmids of pRUM, Inc18 and pheromone plasmids (see sections below). Although some of these mosaic plasmids were detected in both Efm and Efs hosts, species-specific plasmid variants were predominant. We have classified the enterococcal plasmids according to the content in rep/rel/TA systems, and RFLP profiles (Table 1, Figure 2). For the better interpretation of the results, we should keep in mind that members of the most common plasmid families classified in this and other studies as Inc10-like (pRE25, pIP501, pVEF1, pVEF2, pVEF3, pIP816, pEF1, pWZ909) or pRUM-like (pRUM, p3753cB, pST17) exhibit a high degree of modular dissociability or propensity for independent variation and shuffling, and may contain multiple replicons or be devoid of conjugation systems, thus making it very difficult to establish an accurate classification and to trace the origin of certain elements [9,33–40]. See Clewes et al. for a comprehensive updated revision of enterococcal plasmids [9]. In the following sections we will describe vancomycin resistant plasmids of Efm and Efs.

**vnaA plasmids of *E. faecium***

They were classified in two broad groups according to the plasmid replication modules and the background epidemiological context. i) pRUM-like variants (Rep₁/₂/pRUM-like + Rkr/pRUM-like ± Rep₁/₂/pRUM-like + Rep₁/₂/pRUM-like + TAN-fac(0) or TAN-fac(1)). All clones recovered in Portuguese hospitals since the late 90s [22,25,31,32]. While ST6 Efs was widely disseminated in all hospitals (Figure 1 and Figure 2).

ii) pRUM derivatives (Rep₁/₂/pRUM-like + relc/pK11) of variable size (30–120 kb) were detected since the mid 90s from a diversity of clonal backgrounds. pRUM plasmids showing different ClaI-digested DNA RFLP patterns were identified carrying a whole copy of Tn1546 (RFLP_1, RFLP_2, RFLP_20, 30–80 kb), IS1216::Tn1546 (RFLP_8–12, 40–120 kb) or IS1216::Tn1546 (RFLP_3–7, RFLP_13, 50–95 kb). Despite the heterogeneity of plasmid profiles, RFLP_3–6 or RFLP_8–10 shared a variable number of common bands that suggest a relationship among them (see Table 1 and Figure 3 for details about relationships among plasmids). pRUM-like plasmids exhibiting distinct RFLP profiles and carrying different transposon variants were isolated in early and recent isolates of different clonal backgrounds (Figure 2). They include ST190, carrying a 60 kb plasmid RFLP_1 type; ST670 carrying a 85 kb exhibiting a RFLP_4 plasmid type; ST656 carrying a 30 kb plasmid designated as RFLP_20, and ST18, ST132, ST280, carrying different
transposon variants. These results suggest multiple independent acquisitions of pRUM-like plasmids and further rearrangements with other elements, some plasmid variants being efficiently transferred among a diversity of different clones. It is of interest to highlight that epidemic ST18 PFGE types H83 (1996) and H92 (2000) harboured two pRUM-like plasmids. One was the rep17.2/pRUM-like::rel6/pEF1 vancomycin resistant plasmid showing RFLP_2 and RFLP_12 and the other was a 25 kb carrying a rep17.1/pRUM gene and a copy of the Axe-Txe toxin-antitoxin system (rep17.1/pRUM + TAAxe-Txe) identical to the pRUM derivatives described to date (pRUM, p5753cB and pS177) (GenBank accession number GQ900487; Figure 2) [12,38,39] and other vancomycin resistant plasmids circulating at international level (Freitas et al., unpublished data). Diversification in the Rep sequences of these pRUM-like plasmids (homology of 96% at nucleotide level and 95% at protein level) might have resulted in the compatibility with similar (but not identical) plasmids in the same clonal background along extended periods of time.

\( i ) \) Incl8 plasmids and mosaic Inc18-pRUM plasmids. Clonally related ST132 and ST18 Efm isolates from Oporto contained Incl8 plasmids (Rep\(_{17.1/pIP501}\), Rep\(_{2/pRE25/pEF1}\), RFLP_14–15) or mosaic plasmids of Inc18 and pRUM (Rep\(_{2/pRE25/pEF1}\), Rep\(_{17.1/pIP501}\), TAAxe-Txe, rep17.2/pRUM-like + Rel6, RFLP_16–19), all carrying IS\(_{1216}\)-Tn1546 variants. Plasmids showing RFLP types 16–19 were highly similar (5 bands/12 bands in common), RFLP_19 being persistently recovered from clonally related ST132, ST368 and ST369 isolates, collected from hospitalized patients of HSA near by sewage plant and the river Douro from 2001 to 2003. This RFLP_19 has been also identified in a VREfm isolate recovered from swine in 2007 (Tn1546 type ''PP-31'', RFLP_19.1), highlighting the remarkable stability of particular VanA Inc18 plasmids in ensembles of related clones able to spread in different hosts [10]. A diversity of Tn1546::IS1216
Figure 2. Clonal and plasmid diversity among VREFm and VREFs from Portugal. Abbreviations: IS, insertion sequence; Efm, Enterococcus faecium; Efs, Enterococcus faecalis; kb, kilobases; BAPS, Bayesian Analysis of Population Structure; ST, sequence type; CC, clonal complex; rep (replicases); rel (relaxases); TA (toxin-antitoxin system); HUC, Hospital Universitário de Coimbra; HSA, Hospital Santo António; HSJ, Hospital São João; HST, Hospital São Teotônio; HPH, Hospital Pedro Hispano; CHCB, Centro Hospitalar da Cova da Beira; HVR, Hospital S. Pedro; SW, sewage wastewaters; UW, urban wastewaters; R, river; ND, not identified; NI, not determined; UK, unknown. aThe distribution of the different isolates is shown by BAPS subgroups as described [19]. bPFGE types shown in bold represented widespread clones in Portuguese hospitals and/or aquatic surroundings over years. cMost Efm isolates expressed resistance to vancomycin, teicoplanin, erythromycin, ampicillin, ciprofloxacin (92–100%) and to a lesser extent to high levels of kanamycin (65%), gentamicin (41%), streptomycin and tetracycline (28% each). While acm was identified in different CC17 and non-CC17 lineages (76%), esp was detected in CC17 isolates (33%, ST132 and its SLVs ST368, ST369) and hyl was sporadically found (9%, ST18, ST125, ST280 isolates) [25]. Efs isolates (mostly ST6) showed resistance to vancomycin, teicoplanin, erythromycin, ciprofloxacin, high levels of gentamicin and kanamycin (82–100%), tetracycline and chloramphenicol (65% each) and high levels of streptomycin (46%), and mostly contained gelE and agg (90%), cyl (82%) and esp (46%) [26]. dTn1546 designation is based on the results obtained by a PCR assay described by Woodford et al. consisting on the amplification of overlapped fragments covering the whole Tn1546 [68]. Fragments of unexpected length were further analysed by sequencing (this study) [27]. The total rep/rel/TA content of isolates is represented according to its location on plasmids of different size ranges. Rep (normal cells), rel (cells with dots) and TA (cells with diagonal stripes) genes belonging to the same plasmid are represented with the same color and that belonging to the same plasmid family with the same range of colors. The content of VanA plasmids including rep, rel, and TA genes is indicated according to the plasmid type in which they were identified, as well as by the numeric nomenclature used by Jensen et al. [72] for replicases (rep1, rep2, rep9, rep14, rep17, rep18a), given new and consistent designations to replicases non described in reference 72 (rep18b, rep18c, rep20). Relaxases were designated per numerical order as designed by M. V. Francia (unpublished data). Rolling-Circle plasmids are represented in green (rep1/pIP501-like, rel1/pIP501), small-theta replicating plasmids in violet (rep2/pRE25/pEF1, rel9/pCF10, rel12/pCIZ2), Inc18-like plasmids in different red tones (rep3/pIP501, rep4/pRE25/pEF1, rel4/pCF10), TA-repA_N plasmids in different blue tones, pRUM in dark blue (rep7/pRUM, rel7/pRUM, TA-repA_N-TaK), plG1 in turquoise (rep8/pG1), phenomone-responsive plasmids in light blue (rep9/pAD1, rel9/pAD1, rel9/pCF10, pofAD1), and pmT1/pMG1 plasmids in grey (rep22/pHT1, rel22/pHT1). Rep families are named Repxa, where xa indicates the number assigned to different replicase families (e.g. rep1/pIP501, rep2/pRE25/pEF1, rep4/pCF10, rep7/pRUM, rep8/pG1, rep9/pAD1, rep10/pCF10, rep11/pCF10, rep12/pCIZ2, rep13/pCIZ2, rep14/pHT1, rep15/pHT1). The name of the most representative plasmid of the family is also represented for a better follow-up of the results (e.g. rep7/pRUM, rep17 from pRUM and related plasmids p5733cB and p5177; rep1/pIP501, rep1 linked to Inc18 plasmids as pIP501, pIP816 and pRE25; rep9/pAD1, rep9 linked to pCF10, pAD1, pTEF1, pTEF2, pTEF3, pRE99, pMG2200; rep14/pHT1-like, rep14 associated with RCR plasmids pEFN1, pRS42 and/or pr11; rep18/pCF10, rep18 from pCF10; rep19/pCF10, rep19 from both pHT1 and pMG1 plasmids). We further specified the name of different plasmids associated with a given group if necessary. For example, it results helpful for Inc18 family given the number of plasmids containing the same rep gene. These plasmids are increasingly identified among isolates of different origins (e.g. rep2/pRE25/pEF1, rep2 with both pHT1 and pMG1 plasmids). Sequencing identified the different variants within these families (see text). Rep18b, Rep18c, and Rep20 were not included in Jensen’s scheme [72] and the numbers were assigned in this paper following that numeration (rep18b/pB82, rep18c/pCIZ2, rep20/pCIZ2, rep20/pMG1). Rep genes were arbitrarily
vanA plasmids appear in bold rectangles. pCF10. Toxin-antitoxin systems included Axe-Txe from pRUM, and/or pB82 plasmids; Rel 3, pRUM; Rel 5, rel from pAD1, pTEF1, pAM373 and the pathogenicity island of V583; Rel 6, pEF1; Rel 8, pHT. Toxin-antitoxin systems included Axe-Txe from pRUM, os-c2− from Inc18 plasmids and par from pAD1. Genes hybridizing in the same band as vanA plasmids appear in bold rectangles.

doi:10.1371/journal.pone.0060589.g002

Variants containing IS\textsubscript{1216} were mostly located on Inc18 plasmids or on mosaic plasmids Inc18-pRUM or Inc18-pAD1. Most variants contained the IS\textsubscript{1216} at 8839nt of the transposon (PP13, PP17, PP20, PP23, PP30) similarly to other Tn\textsubscript{1546} variants previously described in Europe [42]. Some of them also harboured different insertions corresponding to unknown sequences (X, PP23) or RCR plasmid sequences (PP10) [43] suggesting frequent recombination between acquired genes/plasmids and housekeeping Efm and Efs plasmids (Figure 4). Tn\textsubscript{1546} type D was specifically linked to megaplasmids from CC5 Efm from swine of different continents (Figure 2, Figure 4).

The presence of early plasmids carrying Tn\textsubscript{1546} belonging to different families suggests independent acquisitions of the transposon by pRUM and Inc18 plasmids, which would have been acquired by diverse Efm and Efs populations. Local fixation would be influenced by connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants.

Discussion

This paper shows the local dynamics of Tn\textsubscript{1546}-vanA among \textit{Enterococci} is shaped by horizontal genetic transfer of pRUM and Inc18 plasmids and by recombination-driven evolution of them within and between Efs and Efm clones. The clonal diversity reported in this study has also been observed in areas where the spread of VRE has been documented [44]. Recent retrospective analysis of enterococcal populations suggests that the temporal evolution of the population biology of \textit{Enterococci} is driven by a succession of epidemic waves of enterococcal human specific lineages, Efm ST78 and Efs ST6 emerging in the last decade at global scale similarly to that reported for other pathogens [19,23,24]. In Portugal, the population structure of VRE analysed in this study comprises isolates of main human Efm lineages, ST18 (ST18, ST132) being much more abundant than ST17 (represented by a single isolate of early ST16 lineage) [31], or ST78 (represented by sporadic ST80 and ST656, the first one linked to early VRE outbreaks) [25,29]. It is worthwhile highlighting the recent detection of isolates of another Efm lineage in hospitals of the Oporto area (http://www.mlst.net) as ST117 Efm (ST78 lineage), which would reflect the increasing trend of isolates belonging to the ST78 lineage at international level. However, regional differences in the rates of VRE cannot be fully explained by clonal replacement dynamics since similar enterococcal clones appear widely distributed in areas with high and low rates of VRE (Tedin AM et al., unpublished data). Instead, local conditions, including type and density of hosts, antibiotic usage, and transmission facilities, may influence regional differences in the proportions of VRE, as suggested by mathematical modelling studies on local trends of antibiotic resistance [45,46]. Clones can locally evolve by variation, drift and short-distance migration, leading to changes in colonization ability, pathogenicity or even host range, the fittest clonal variants being able to facilitate the spread of antibiotic resistance [23,47–50]. The observed clonal heterogeneity of the predominant ST18 lineage which comprises particular ST18 and ST132 strains widespread in different cities, highlights the role of certain efficiently transmissible clones in the
Table 1. Plasmids identified in this study.

| RFLP type | VanA modular profile | Size | No. isolates | Tn1546 | PFGE type | City | Year |
|-----------|----------------------|------|--------------|--------|-----------|------|------|
| RFLP_1    | Rep17.2::Rel6         | 60   | 1            | A      | ST190_H98 | Coimbra | 1998 |
| RFLP_2    | Rep17.2::Rel6         | 80   | 1            | A      | ST18_H92 | Coimbra | 2000 |
| RFLP_8    | Rep17.2::Rel6         | 120  | 1            | PP2b   | ST18_H70 | Coimbra | 2001 |
| RFLP_9    | Rep17.2::Rel6         | 60   | 1            | PP2b   | ST18_H70 | Viseu | 2002 |
| RFLP_10   | Rep17.2::Rel6         | 60   | 3            | PP2b   | ST18_H70, H93 | Coimbra, Viseu | 2002 |
| RFLP_11   | Rep17.2::Rel6         | 50   | 1            | PP5    | ST18_H87 | Coimbra | 2002 |
| RFLP_6    | Rep17.2::Rel6         | 80   | 1            | PP5    | ST18_H70 | Viseu | 2003 |
| RFLP_5    | Rep17.2::Rel6         | 90   | 12           | PP2, PP4, PP5, PP24 | ST18_H78, H72, H94, H123, H129 | Coimbra, Porto, Matosinhos | 2001–2007 |
| RFLP_3    | Rep17.2::Rel6         | 95   | 3            | PP4    | ST18_H108 | Coimbra | 1998–2000-NI |
| RFLP_4    | Rep17.2::Rel6         | 85   | 2            | PP4    | ST670_H90, ST18_H81 | Coimbra | 1997–2001 |
| RFLP_7    | Rep17.2::Rel6         | 50   | 1            | PP5    | ST18_H70 | Viseu | 2008 |
| RFLP_12   | Rep17.2::Rel6         | 40   | 1            | PP5    | ST125_H126 | Porto, Viseu | 2002–2003 |
| RFLP_13   | Rep17.2::Rel6         | 40   | 1            | PP5    | ST280_H100 | Vila Real | 2008 |
| RFLP_18   | Rep17.2::Rel6         | 60   | 1            | PP5    | ST125_H126 | Matosinhos | 2007 |
| RFLP_19   | Rep17.2::Rel6         | 60   | 3            | PP5    | ST18_H25 | Viseu | 2009 |
| RFLP_20   | Rep17.2::Rep2::Rel6   | 30   | 1            | PP5    | ST656_H130 | Porto | 2001 |
| RFLP_14   | Rep17.2::Rep2::Rel6   | 40   | 1            | PP5    | ST132_H119 | Porto, Viseu | 2002 |
| RFLP_15   | Rep17.2::Rep2::Rel6   | 60   | 2            | PP5    | ST18_H72 | Porto, Viseu | 2003 |
| RFLP_21   | Rep17.2::Rep2::Rel6   | 75   | 1            | PP5    | ST18_H125 | Viseu | 2004 |
| RFLP_22   | Rep17.2::Rep2::Rel6   | 85   | 1            | PP5    | ST366_H99 | Coimbra | 2000 |
| RFLP_23   | Rep17.2::Rep2::Rel6   | 90   | 12           | PP3, PP4, PP5, PP24 | ST18_H78, H72, H94, H123, H129 | Coimbra, Porto, Matosinhos | 2001–2007 |
| RFLP_24   | Rep17.2::Rep2::Rel6   | 90   | 1            | PP5    | ST132_H123 | Coimbra | 2001 |
| RFLP_25   | Rep17.2::Rep2::Rel6   | 50   | 1            | PP5    | ST366_H99 | Porto, Viseu | 2002 |
| RFLP_26   | Rep17.2::Rep2::Rel6   | 60   | 1            | PP5    | ST280_H100 | Porto, Viseu | 2002–2003 |
| RFLP_27   | Rep17.2::Rep2::Rel6   | 60   | 1            | PP5    | ST125_H126 | Porto, Viseu | 2007 |
| RFLP_28   | Rep17.2::Rep2::Rel6   | 75   | 1            | PP5    | ST125_H126 | Matosinhos | 2007 |
| RFLP_29   | Rep17.2::Rep2::Rel6   | 85   | 1            | PP5    | ST125_H126 | Porto, Viseu | 2001 |
| RFLP_30   | Rep17.2::Rep2::Rel6   | 90   | 1            | PP5    | ST125_H126 | Porto, Viseu | 2002 |
| RFLP_31   | Rep17.2::Rep2::Rel6   | 100  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2003 |
| RFLP_32   | Rep17.2::Rep2::Rel6   | 110  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2004 |
| RFLP_33   | Rep17.2::Rep2::Rel6   | 120  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2005 |
| RFLP_34   | Rep17.2::Rep2::Rel6   | 130  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2006 |
| RFLP_35   | Rep17.2::Rep2::Rel6   | 140  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2007 |
| RFLP_36   | Rep17.2::Rep2::Rel6   | 150  | 1            | PP5    | ST125_H126 | Porto, Viseu | 2008 |

Abbreviations: RFLP, restriction fragment length polymorphism; ST, sequence type; NI, not identified.

*Plasmid type RFLP_12 (Rep17.2/pRUM-like + Rep2/pRE25/pEF1 + Rel6/pEF1) contains a partial sequence of the replication gene of the RCR plasmid pEFNP1 (GenBank accession number AB038522), suggesting the integration of this RCR plasmid on the mobile element carrying Tn1546 involving truncation of the rep2/pRUM-like + Rep2/pRE25/pEF1.

**Plasmid types RFLP_3, 5, 6, 11 (Rep17.2/pRUM-like + Rel6/pEF1 and eventually containing Rep1/pIP501, Rep2/pRE25/pEF1 or TAInc18) shared common bands and were identified in the same or different clonal backgrounds in different cities for extended periods of time.

***Plasmids showing patterns related to RFLP_27 (75–85 kb; rep9/pAD1 + rel5/pAD1 + par/pAD1 and/or rep2/pRE25/pEF1) initially recovered from the widespread ST6-CC2 Efs clone in Coimbra in 1996 and other Efs (ST55 and ST159) and Efm clones contained similar IS1216-Tn1546 variants (PP-2a, PP-4, PP-9). Other highly related mosaic Inc18-pAD1-related plasmids carrying IS1216-Tn1546 were recovered from ST6 VREfs and ST80 VREfm isolates type IIEfs, rep9/pAD1 + rep2/pRE25/pEF1 versus type IIEfm, rep9/pAD1 + rep2/pRE25/pEF1.

doi:10.1371/journal.pone.0060589.t001
dissemination of antibiotic resistance. Successful clones can eventually be able to disseminate at international level as strains of ST6 Efs or ST280 Efm within main Efm human lineages driving or contributing the spread of different traits as Tn1546 or Tn1549 [51]. One remarkable fact is the similarity among PFGE patterns of isolates with different STs. Given the high content of plasmids and transposons of the isolates studied, and the frequent rearrangements identified among Efm and/or Efs isolates [21], chromosomal transfer cannot be discarded. Recent phylogenomic analysis based on the degree of admixture among a diversity of isolates studied suggests that recombination is restricted to isolates within specific BAPS groups [19]. Most plasmids coding for vancomycin resistance are found in similar clonal backgrounds. This observation suggests that recombination does occur within isolates of similar BAPS groups as recently described [19]. However, the observed mosaicism and enhanced host range of particular plasmid variants indicates the existence of an unexpectedly high degree of connectivity between phylogenetically distant enterococcal populations and/or in bacterial genetic exchange communities integrating enterococci.

Broad host and narrow host plasmids carrying vancomycin resistance would have a high “betweenness centrality”, which is a pivotal index in network theory useful for measuring the load placed on the given node in the network as well as the node’s importance to the network than just connectivity [52]. A recent in silico network analysis of all plasmid sequences available at the GenBank databases confirms very high betweenness values for some Inc18 plasmids as pVEF3 (an Inc18 derivative highly spread among Efm from animals in Europe) [13,37], and also for a pheromone-responsive plasmid pTEF1 (a plasmid recovered from ST6_Efs strain V583, highly related to the ST6 described in this work) [33] (unpublished data). Other plasmids with a high degree of modular dissociability, would be pRUM-like elements, which may enhance their complexity resulting in new configurations with enhanced betweenness. It is tempting to suggest that plasmid variability has contributed to intra-clonal diversification both in Efm and Efs, giving rise to a local wealth of clonal variants able to fully explore the local adaptive landscape. In fact, this and other studies demonstrate that selected variants of Inc18, pAD1, and pRUM plasmids can determine differences in the dynamics of VRE in different areas, further influencing the plasmid host range and the selection of specific clones within human adapted lineages. Examples of widespread plasmid variants of Inc18 or pRUM plasmids coding for vancomycin resistance have been reported recently. They included Inc18 widespread among Efm poultry isolates from Europe [13] or among Efs clinical isolates from the USA, the last one being able to transfer Tn1546 to S. aureus [15]; and mosaics of pRUM variants containing Axe-Txe and Inc18 from humans in different continents (Freitas AR et al. unpublished data). The identification of chimeric pRUM-Inc18 plasmids containing rep/rel/TA of Inc18 sequences and Tn1546 variants widely observed in poultry, hospitals and hospital sewage in the Oporto area reflects genetic exchanges between enterococci from different origins and highlights the need to enforce barriers to avoid the spread of multidrug resistance human pathogens to the environment and viceversa.

In this scenario, the genetic context of Tn1546 seems to greatly influence the evolvability of the transposon and explains the high diversity of variants found in this and other studies [1,27,42,54]. The frequent presence of insertions in the backbone of Tn1546 and the abundance of IS1216 and ISEf1 in enterococcal genomes [9,55] makes homoplastic evolution of Tn1546 in different backgrounds possible. However, other IS (IS1251, IS1542, IS1476, IS19 and IS1405) linked to different plasmid and clonal backgrounds [9,40] have been identified at different sites of Tn1546, thus suggesting that chance and selection are responsible to differences in variants collected in different areas. The widespread of Inc18 plasmids with a common origin in Europe [13,56] indicates local fixation of Tn1546 influenced by a founder effect and further connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants as reported in this study.

Our results suggest that VRE spread is facilitated by selected clones of different lineages through strong interactive processes of clonalization and plasmid diversification that might occur at local scales. Despite the maintenance of significant gene flow, a sympatric, or more probably, parapatric bacterial clonalization process (when diverging populations share a common or neighbouring environment), might contribute to the formation of temporary genetic mosaics and the preservation of ecologically important genomic traits [57]. Such micro-evolutionary process will result in an array of clonal complexes forming a population structure able to exploit the local spatio-temporal patch heterogeneities [58]. Note that exploitation of connected microenvironments should accelerate evolution of antibiotic resistance [59]. The expected result of such a successful population structure is the local persistence of antibiotic resistant clones, and eventually the local fixation [60] of vancomycin-resistance [46].

In summary, this study highlights the relevance of studying the local microecology of genes, elements, lineages and populations to decipher the robustness of the trans-hierarchical networks connecting these evolutionary elements in order to describe and predict the local evolvability of vancomycin-resistance [61]. Traditional surveillance studies are one-off cross sectional surveys focused on single traits as epidemic strains, genes or mobile genetic elements over limited periods of time which only gives one shot view that precludes addressing the long-term dynamics of antibiotic resistance. The more comprehensive approach described in this study is needed for understanding in depth the evolution of complexity in multihierarchical systems as those involved in the
spread of antibiotic resistance among the populations of bacterial human pathogens.

Materials and Methods

Bacterial strains and epidemiological background

One hundred four VRE clinical isolates carrying Tn1546 from different regions of Portugal, 75 VREfm and 29 VREfs, were analyzed in this study. They included: i) clinical isolates from hospitals of Coimbra (Hospital Universitário de Coimbra, HUC), Oporto (Hospital Santo António, HSA), Viseu (Hospital de São Troímeno, HST); Matosinhos (Hospital Pedro Hispano, HPH), Vila Real (Hospital S. Pedro, HVR) and Covilhã (Centro Hospitalar da Cova da Beira, CHCB) located in Northern and Central Portugal (62 Efm and 26 Efs; 1996–2008); ii) isolates from waste waters of hospitals (HSA and Hospital de São João, HJS) (10 Efm and 3 Efs), and iii) isolates from the estuary of the River Douro (3 Efm) recovered in the Oporto area during 2001–2003. Part of the isolates analyzed in this work corresponds to strains from previous surveillance studies [25–27,62]; this paper constitutes the first description of isolates obtained during 2007 and 2008. Contemporary Portuguese VRE isolates of animal origin were used for comparative analysis of lateral transfer events [10].

Susceptibility against 15 antibiotics was determined by the agar dilution method following CLSI standard guidelines. Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE), banding patterns were interpreted according to criteria previously suggested for long-term studies, and multilocus sequence typing (MLST) as described elsewhere (http://efaecium.mlst.net) [25,63–65].

The presence of putative virulence traits [collagen-binding adhesin (cwa), enterococcal surface protein (esp), hyaluronidase (hyp), cytolysin/hemolysin (cyt), gelatinase (gel) and aggregation substance (agg)] was searched for using PCR as described [66,67].

Genetic context of Tn1546

Characterization of Tn1546 backbone was determined by amplification of overlapping transposon fragments and further sequencing of PCR products [27,66]. We have accomplished the analysis for the isolates not studied in previous surveys and have interpreted the resulting transposon diversity (this study) [27], under the light of the plasmid and clonal backgrounds identified in this geographical area.

Plasmid analysis

Isolates (n = 62 Efm and n = 15 Efs) representing the clonal diversity observed in both species were selected for plasmid characterization (Table 1, Figure 2). The content and size of plasmids from transconjugants obtained by filter mating were determined by using either the technique described by Barton et al. (plasmids >10 kb) or the alkaline lysis extraction method of Kado & Liu (plasmids <10 kb) [51,69,70]. Classification of E. faecium plasmids was based on the presence of specific modules for replication (rep-initiator proteins), mobilization (relaxases) and stability (toxin-antitoxin systems). Relaxases (rel) were sought by a multiplex-PCR-based relaxase typing method which differentiates relaxases of the MOBQ, MOBF, MOBC and MOBV families related to 27 known plasmids [9,71] (Francia MV, unpublished data). Replication initiator proteins (rep) were investigated by amplification of 24 replicons, which allows discriminating among DNA sequences from more than 100 published Gram-positive plasmids [9,72]. Designation of rep sequences pointed out the plasmid type in which they were initially identified, as well as the numeric nomenclature originally used by Jensen et al. (Figure 2s footnote) [72]. Toxin-antitoxin systems (TA) previously identified among streptococci and enterococci (Axe-Tx, e-c-zl par, mar/EF) or Gram-negative bacteria (relBE) were detected by PCR [73]. PCR products were sequenced in order to confirm the specificity of the method and to analyze similarities with other well-characterized plasmids. Genomic location of the Tn1546 and the rel/rep/TA sequences was determined by hybridization of vanA and rel/rep/TA specific probes obtained by PCR from DNA reference plasmids with S1 or L-Cool digested genomic DNA from representative strains [51,69]. Structural relationship between plasmids of similar size was established by comparison of their RFLP patterns obtained after digestion with different restriction enzymes (EcoRI, HindIII and CiaI; see Figure 3). Plasmid DNA was obtained by using a modified protocol based on the alkaline lysis method described by Handwerger et al. [74] consisting of increasing two-fold the volume of lysosome, SDS/NaOH and acetate potassium solutions, extending the incubation period in potassium acetate solution for at least three hours, precipitating the supernatant obtained after extraction with phenol-chloroform using ethanol-acetate potassium solution (20:1 vol/vol) at 25°C for at least 2 hours, and resuspending final DNA pellets in 30 μl of water for further enzyme digestion analysis.

Molecular techniques

Southern blot DNA transfer and hybridization were performed by standard procedures [75]. The vanA and rep/rel/TA/bac probes used in the hybridization assays were generated by PCR using well known positive controls as template DNA. Labelling and detection were carried out using Gene Images Alkphos Direct Labelling system kit, following the manufacturer’s instructions (Amersham GB/GE Healthcare Life Sciences UK Limited). PFGE was performed as described previously [76] using the
following conditions: switch time of 5 s to 25 s for 6 h, followed by 30 s to 45 s for 10 h (S1 nuclease); 5 s to 30 s for 22 h, 14°C, and 6 V/cm² (I-Cut) and 1 s to 20 s for 26 h, 14°C, and 6 V/cm² (Smad).

Plasmid sequences

Analysis of nucleotide and aminoacid sequences revealed two types of sequences amplified with primers used for identification of repT1/pRUM. They were 100% (designated as RepT1/pRUM) or 97% (96% identity at amino acid level; designated as RepT1/pRUM-0.5k) homologous to those of RepA_pRUM (GenBank accession number AF50797.7). Most RepT1/pRUM-0.5k aminoacid sequences were 98%-100% identical to RepE_pPI501, a member of the Inc18 family (GenBank accession number AM932524), and to a lesser degree to pRE25, pTEF1 or pSM19035; and Rep2/pRE25/pEF1 showed 96%-100% amino acid identity to that of pEF1 (GenBank acc. no. DQ198089). Sequences identified as Rel6/pEF1 showed 98%-100% homology to orf34_pEF1. Relaxases of the E. faecalis pheromone-responsive plasmids identified in this study displayed a high homology with those of known enterococcal pheromone plasmids pAD1, pAM373 and pTEF1 (orf17_pAD1, GenBank acc. no. AAL59457; EFA0025_pTEF1, GenBank AE016833; and EP0019_pAM373, GenBank acc. no. NC_002630). That of plasmid showing RFLP_27 showed a 67%-84% homology with the above mentioned pheromone enterococcal plasmids but 94% identity with a MobC relaxase (annotated as a hypothetical protein) from a vancomycin-resistant S. aureus strain (GenBank acc. no. EIK35827).

Acknowledgments

We are grateful to the personal involved in the gift of the strains from the different Portuguese healthcare institutions: HSA (Hospital Santo António, Porto), HPH (Hospital Pedro Hispano, Matosinhos), CHCB (Centro Hospitalar da Cora da Beira, Covilhã), HST (Hospital S. Teotónio, Viseu), and HVR (Hospital S. Pedro, Vila Real). We are grateful to Lars B. Jensen, Eva Sadowy, Arinmuni Sundsfjord, Guido Werner, and Rob Willems for providing suitable control cultures.

Author Contributions

Performed most experimental work: ARF. Partially contributed to clonal and transposon characterization: CN. APT. Conceived and designed the experiments: ARF LP TM. Analyzed the data: ARF CN AN MVF LP TM. Contributed reagents/materials/analysis tools: ARF LP TM. Wrote the paper: ARF LP TM.

References

1. Bonten MJM, Willems RJ, Weinstein RA (2001) Vancomycin resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis 1: 313-323.
2. Murray BE (2000) Vancomycin-resistant enterococcal infections. N Engl J Med 342: 710-721.
3. Verner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, et al. (2000) Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 13: pii 19046.
4. De Lencastre H, Brown AE, Chung M, Armstrong D, Tomasz A (1999) Role of the transposon Tn552 in the epidemiology of vancomycin-resistant Enterococcus faecium in the pediatric oncology unit of a New York City hospital. Microb Drug Resist 5: 113-129.
5. Martone WJ (1998) Spread of vancomycin-resistant enterococci: why did it happen in the United States? Infect Control Hosp Epidemiol 19: 339-345.
6. Thal L, Donabedian S, Robinson-Dunn B, Chow JW, Dembry L, et al. (1998) Molecular analysis of glycopeptide-resistant Enterococcus faecium isolates collected from Michigan hospitals over a 6-year period. J Clin Microbiol 36: 3303-3308.
7. Kaswalde M, Guadickowski M, Hryniewicz W (2000) Outbreak of vancomycin-resistant enterococci in a hospital in Gdansk, Poland, due to horizontal transfer of different Tn554-like transposants variant and clonal spread of several strains. J Clin Microbiol 38: 3317-3322.
8. Stampone L, Del Grosso M, Boccia D, Pantosti A (2005) Clonal spread of a vancomycin-resistant Enterococcus faecium strain among bloodstream-infecting isolates in Italy. J Clin Microbiol 43: 1475-1500.
9. Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, et al. (2013) Extrachromosomal and Mobile Elements in Enterococci. Transmission, Maintenance, and Epidemiology. In: Clewell DB, Shankar N, Ike Y, and H.marsh (Eds.) Extrachromosomal Elements in Bacteria: their Role in Genetic Exchange. Elsevier Science B.V.
10. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L (2009) Dispersion and HVR (Hospital S. Pedro, Vila Real). We are grateful to Lars B. Jensen, Eva Sadowy, Arinmuni Sundsfjord, Guido Werner, and Rob Willems for providing suitable control cultures.

Plasmids and VRE Clonization
32. Willems RJ, van Schaik W (2009) Transition of Enterococcus faecium from commensal organism to nosocomial pathogen. Future Microbiol 4:1125-1135.
33. Weaver KE, Kwong SM, Firth N, Francia MV (2009) The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. Plasmid 61: 94-109.
34. Schwartz FV, Petre M, Teuber M (2001) Sequence of the 56kb conjugative multiresistance plasmid pRE25 from Enterococcus faecalisRE25. Plasmid 46: 170-187.
35. Rieck-Barcha JL, Flaviano B, Maldonado-Barragán A, Jiménez-Diaz R (2007) Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from Enterococcus faecium 6T1a. Plasmid 57: 175-181.
36. Stletvod H, Johansen PJJ, Simonsen GS, Asaanes B, Sundsfjord A, et al. (2007) Comparative analysis of two 58-kb plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway. Antimicrob Agents Chemother 51: 736-739.
37. Grady R, Hayes F (2003) Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium. Mol Microbiol 47: 1419-1432.
38. Halvorsen EM, Williams JJ, Bhamani AJ, Billings EA, Hergenrother PJ (2011) Txe, an endoendonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mcrNA and inhibits protein synthesis. Microbiology 157: 387-397.
39. Coque TM, Freitas AR, Nocais C, Pereira L, Baquero F (2012) Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci. In: Enterococcus and Safety. Series: Advances in Food Safety and Food Microbiology. Semedo-Lemsaddek T, Barreto-Crespo MT, Tenreiro R (eds). Nova Publishers. ISBN: 978-1-61470-392-9.
40. Soren M, Johansen PJJ, Jnomes B, Rosovii T, Kruse H, et al. (2006) Prevalence, persistence, and molecular characterization of glycopenptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. Appl Environ Microbiol 72: 516-521.
41. Schouten MA, Willems RJ, Kraak WA, Top J, Hoogkamp-Korstanje JA, et al. (2001) Molecular analysis of Tn1546-like elements in vancomycin-resistant enterococci isolated from patients in Europe shows geographic transposon type clustering. Antimicrob Agents Chemother 45: 986-989.
42. Garcia-Migura L, Hasman H, Jensen LB (2009) Presence of pRI1: a small plasmid-encoded in vancomycin-resistant enterococci from humans and animal origin. Curr Microbiol 58: 95-100.
43. Zheng B, Tomita H, Xiao YH, Wang S, Li Y, et al. (2007) Molecular characterization of vancomycin-resistant Enterococcus faecium isolates from mainland China. J Clin Microbiol 45: 2813-2818.
44. McCormack AW, Whitney CG, Farley MM, Lynfield R, Harrison LH, et al. (2003) Geographic diversity and temporal trends of antimicrobial resistance in Staphylococcus pneumoniae in the United States. Nat Med 9: 424-430.
45. Johansen PJJ, Townsend JP, Bohn T, Simonsen GS, Sundsfjord A, et al. (2009) Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect Dis 9: 357-364.
46. Price LB, Stegger M, Hasman H, Aziz M, Larsen JS, et al. (2012) Staphylococcus aureus CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. MBio 3: pii: e00305-11.
47. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, et al. (2005) Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone. Lancet 365: 1256-1258.
48. de Regt MJ, van Schaik W, van Luit-Asbroek M, Dekker HA, van Duijkeren E, et al. (2012) Hospital and community ampicillin-resistant Staphylococcus aureus: Host Adaptation and Emergence of Methicillin Resistance in 741. Garcillan-Barcia MP, Francia MV, de la Cruz F (2009) The diversity of vancomycin-resistant Enterococcus faecium and criteria for defining strains. J Clin Microbiol 37: 1094-1091.
49. Narrapareddy SR, Weinstock GM, Murray BE (2003) Clinical isolates of Enterococcus faecium exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol 47: 1733-1747.
50. Vankerckhoven V, van Autgaerden T, Vael G, Lammens C, Chapelle S, et al. (2004) Development of a multiplex PCR for the detection of tsa, gelE, cld, esp and ldy genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol 42: 4473-4479.
51. Woodford N, Adebiyi AMA, Palepou MFI, Cookson BD (1998) Diversity of vanA glycopeptide-resistant enterococci in humans and nonhuman sources. Antimicrob Agents Chemother 42: 502-508.
52. Gordon DM, Woodford N, Barrett SP, Sussan P, Cookson BD (1999) DNA banding pattern polymorphism in vancomycin-resistant Enterococcus faecium and criteria for defining strains. J Clin Microbiol 37: 1094-1091.
53. Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365-1373.
54. Garcia-Barriga MP, Francia MV, de la Cruz F (2009) The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol Rev 33: 657-687.
55. Jensen LB, Garcia-Migura L, Valenzuela JA, Lohr M, Hasman H, et al. (2010) A classification system for plasmids from enterococci and other Gram-positive bacteria. J Microbiol Methods 80: 25-43.
56. Moritz EM, Hergenrother PJ (2007) Toxin-antitoxin systems are ubiquitous and evolutionarily closely linked but have diversified through niche adaptation. PLoS Biol 5: e30319.