Interkingdom Transfer of the Acne-Causing Agent, *Propionibacterium acnes*, from Human to Grapevine

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Abstract

Here, we report the surprising and, to our knowledge, unique example of horizontal interkingdom transfer of a human opportunistic pathogen (*Propionibacterium acnes*) to a crop plant (the domesticated grapevine *Vitis vinifera* L.). Humans, like most organisms, have established a long-lasting cohabitation with a variety of microbes, including pathogens and gut-associated bacteria. Studies which have investigated the dynamics of such associations revealed numerous cases of bacterial host switches from domestic animals to humans. Much less is, however, known about the exchange of microbial symbionts between humans and plants. Fluorescent in situ hybridization localized *P. acnes* in the bark, in xylem fibers, and, more interestingly, inside pith tissues. Phylogenetic and population genetic analyses suggest that the establishment of the grapevine-associated *P. acnes* as obligate endophyte is compatible with a recent transfer event, likely during the Neolithic, when grapevine was domesticated.

Key words: **symbiosis, pathogen, endophyte, *Propionibacterium acnes*, *recA*.**

Introduction

Bacteria are highly adaptable microorganisms routinely found as symbionts or pathogens on plants and animals alike. Some human bacterial pathogens are even able to colonize plant tissues (Tyler and Triplett 2008), a phenomenon that in most cases can be considered as an opportunistic exploitation of a temporary habitat. For example, some microorganisms have a multihost life cycle and may use plants as alternative/intermediate hosts, as in the case of enteric human pathogens *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* (Cooley et al. 2003; Kutter et al. 2006; Bernstein et al. 2007; Lacava et al. 2007). Notably, most bacterial endophytes, including the aforementioned human pathogens, are facultative endophytes, which colonize plant tissues only during part of their life time (Klerks et al. 2007). In contrast, obligate endophytes exclusively colonize plants and therefore need to be adapted to the host environment.

Although exploiting plants as temporary hosts may be common, host switch followed by an adaptation to the new host is relatively rare and occurs preferentially between closely related hosts such between insects (Russell and Moran 2005) or vertebrates (Walter et al. 2011). Here, we report a striking case of interkingdom bacterial host transfer involving the human pathogen *Propionibacterium acnes* and grapevine plants (*Vitis vinifera* L.). *Propionibacterium acnes* is the causing agent of acnes and is generally associated with human skin, where it feeds on fatty acids secreted by sebaceous glands (Webster et al. 1981; Zouboulis 2004). This species is also present in the human gastrointestinal tract (Perry and Lambert 2011), but it may exhibit opportunistic pathogenicity by causing postsurgical infection (Nisbet et al. 2007) and has also been occasionally found in other animals (Webster et al. 1981; Lyons et al. 2009). We hereby report a new type of *P. acnes* characterized by an endophytic lifestyle in grapevine and which we suggest has adapted to the new host to the point of obligate endophytism and possibly endocellular symbiosis. We propose to name this *P. acnes* type Zappae (hereafter named *P.* Zappae) (Named after the Italian term "zappa" [hoe] as well as a tribute to eccentric composer Frank Zappa, to highlight the unexpected and unconventional habitat of this *P. acnes* type.)

We first discovered the endophytic habitat of *P.* Zappae while performing a 16S rDNA survey of the grapevine endomicrobiome. Specifically, Roche 454 amplicon sequencing of the V5–V9 16S rDNA hypervariable region resulted in ~6,800 sequences ascribable to *P. acnes*-like bacteria. These sequences could be assigned to 54 of the 60 analyzed plants and accounted for 0.5% to 5% of the total endophytic bacterial microbiota.

The consistent detection after the accurate peeling and sterilizing process adopted strongly suggests that *P.* Zappae is endophytic. This finding is confirmed by fluorescence in situ hybridization (FISH) microscopy observations that made

Letter

Fast Track

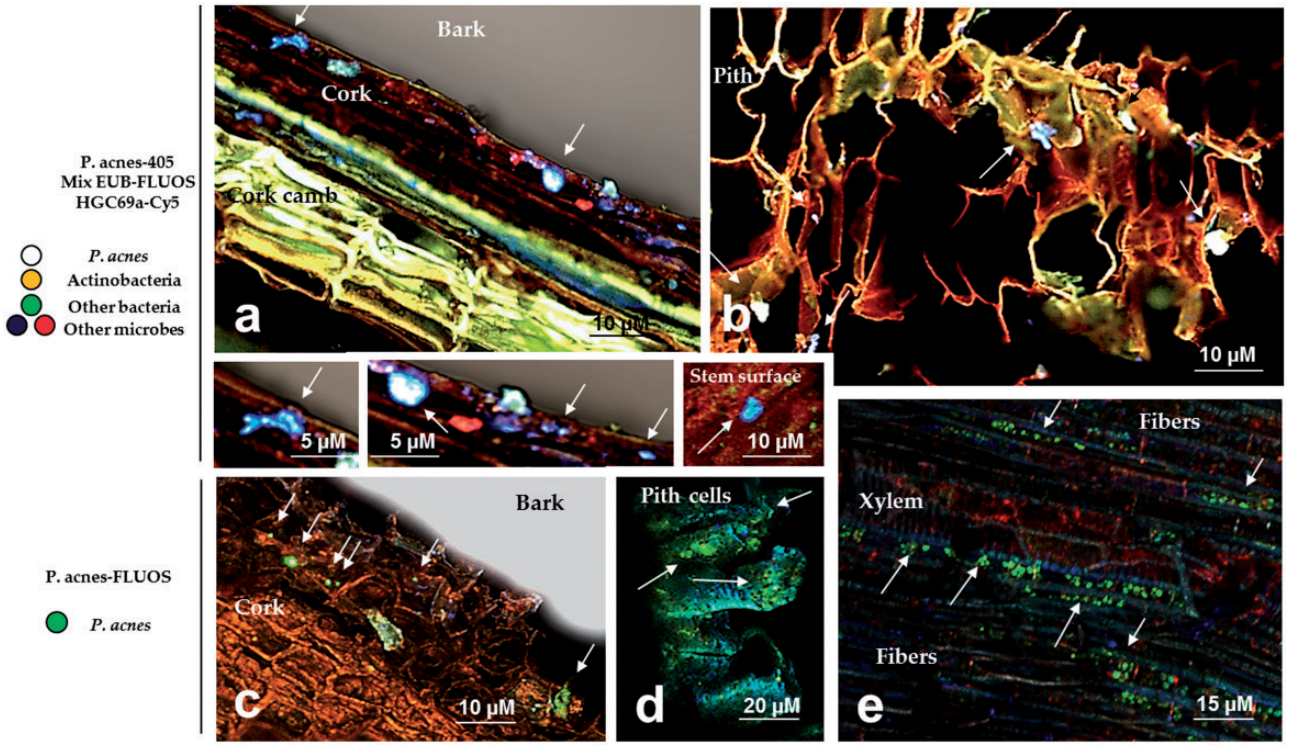

Fig. 1. Localization of *Propionibacterium acnes* type Zappae in grapevine. CSLM microphotographs of grapevine stems showing presence of *P. acnes* (*a*) in bark and (*b*) in pith. Arrows point at white/low blue fluorescent *P. acnes* due to the use of *P. acnes* probe-405 dylight (blue channel), EUBmix-FLUOS (green channel), HGC69a-Cy5 probe (red channel); RGB channels were merged using ImageJ; (*c*) localization of *P. acnes* in bark and (*d*) in pith cells; (*e*) *P. acnes* occasional detection near xylem in fibers. In (*c*–*e*), *P. acnes* probe coupled with FLUOS was used to show *P. acnes* as green fluorescent instead of white fluorescent.

use of different fluorochromes and probes (fig. 1; supplementary figs. S1 and S2, Supplementary Material online). In particular, FISH revealed that this bacterium colonizes bark tissues (fig. 1*a* and *c*), occasionally fibers of xylem vessels (fig. 1*e*), and, more interestingly, pith (fig. 1*b* and *d*), where the bacterium might be intracellular (fig. 1*d*). Notably, FISH also revealed that only a minority of the other endophytic bacteria reside in the pith, whereas the majority, including actinomycetes, were found in the vascular tissues, parenchyma, and fibers surrounding xylem vessels (supplementary figs. S1 and S2, Supplementary Material online). The specific localization of *P.* Zappae within plant tissues suggests that, while this bacterium has retained its anaerobic habit, it is subject to distinct selective pressures compared with its human counterpart.

To investigate the evolutionary history of *P.* Zappae, we performed a phylogenetic analysis of the 16S rDNA gene by randomly sampling one representative sequence from each of the 54 grapevine samples. The resulting maximum likelihood tree showed that *P.* Zappae 16S rDNA gene sequences cluster in a distinct group, and are closely related to *P. acnes* extracted from public databases (fig. 2*a*). To confirm the affinity between *P. acnes* and *P.* Zappae, we further amplified two widely used species-specific *P. acnes* marker genes, *recA* and *tly*, from total DNA isolated from 12 of the tested plants (Rossi et al. 1999; McDowell et al. 2008; Sfanos and Isaacs 2008; McDowell et al. 2012). Phylogenetic inferences based on these two genes revealed that *P.* Zappae *recA* and *tly* sequences are mostly nested within the clade containing *P. acnes* type I (fig. 2*b* and *c*; supplementary fig. S3, Supplementary Material online; for a description of *P. acnes* population structure see McDowell et al. [2008]; note that more clones were amplified from each plant, for a total of 51 sequences per gene). Because all of the three phylogenies of figure 2 are rooted using *P. avidum*, which like *P. acnes* is found mainly on humans, our results support a human origin for the endophytic *P.* Zappae. The inconsistency between the presence of two distinct clusters, one for *P.* Zappae and one for *P. acnes*, in the 16S rDNA gene phylogeny compared with the interspersed *P.* Zappae *recA* and *tly* sequences among those of *P. acnes* suggests incomplete lineage sorting, a typical signature of very recent speciation/diversification (Maddison 1997). Comparison of the three phylogenies further revealed gene-specific patterns of evolution. For instance, in *P.* Zappae, *recA* accumulates many more mutations than *tly* and the 16S rDNA gene of *P.* Zappae (compare red branches in fig. 2). Second, the starlike shape of *P.* Zappae *recA* phylogeny (fig. 2*a*, see also alignment in supplementary fig. S4, Supplementary Material online) indicates that multiple distinct isolates colonize grapevine.

To better understand the forces behind the fast evolution of *recA* in *P.* Zappae, we performed population genetic analyses of this gene and the putatively neutrally evolving genes *tly* and 16S. The *recA* gene is highly polymorphic in the endophytic *P.* Zappae (table 1; supplementary fig. S4, Supplementary Material online), with rare variants

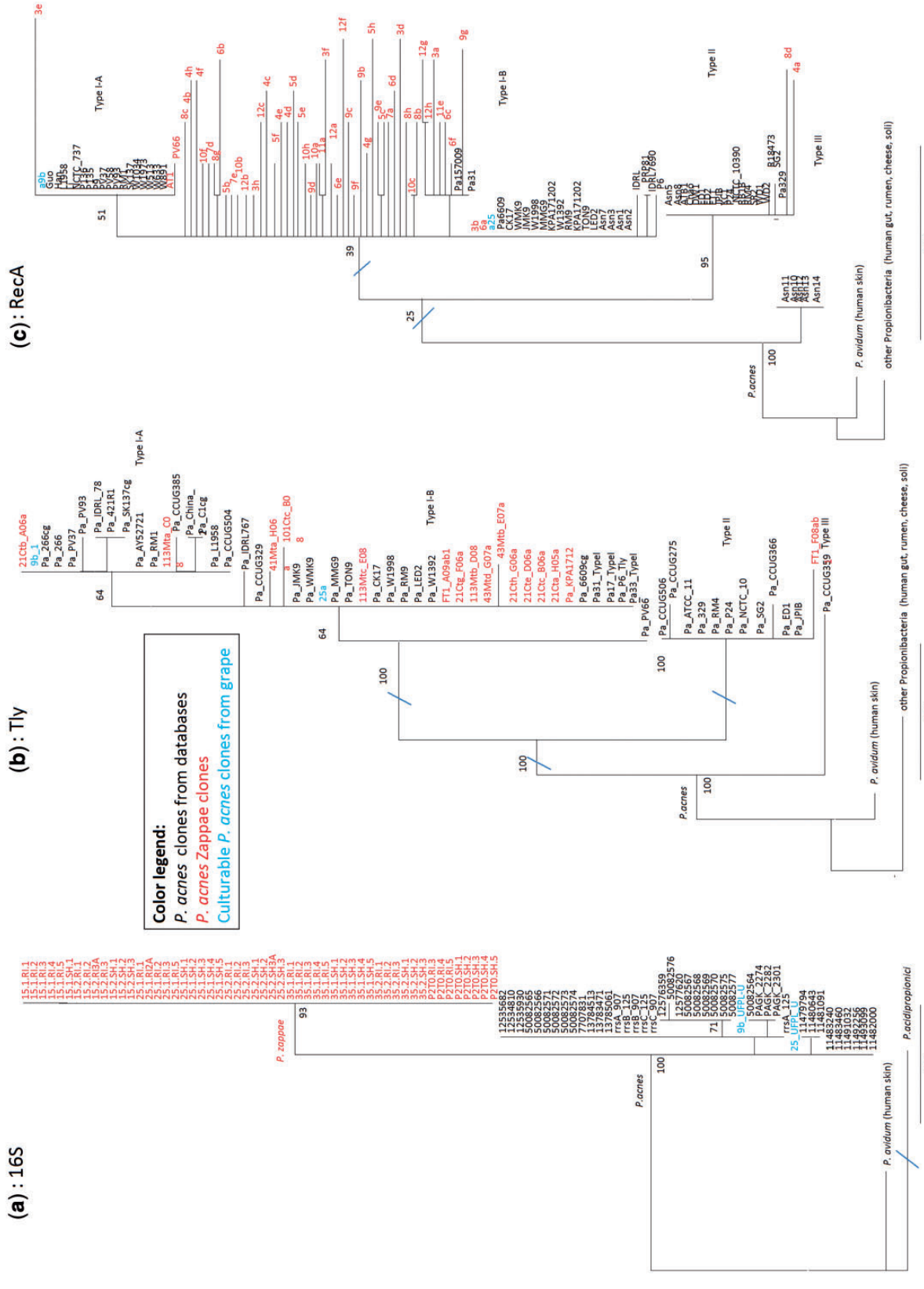

Fig. 2. Phylogeny of *Propionibacterium* Zappae according to 16S rDNA gene (*a*), *tly* (*b*), and *recA* (*c*) markers. Grape endophytic *P.* Zappae (in red) clusters close or within *P. acnes* depending on the marker. According to *tly* and *recA* they belong to subgroup type I and II of the common *P. acnes* normally found in humans (black text corresponds to entries downloaded from GeneBank). *RecA* accumulates more and different types of mutations in endophytic *P.* Zappae than in human associated *P. acnes*. Supports at nodes are maximum likelihood bootstrap supports. Other propionibacteria were used to root the tree, but their branches are not always in scale; the complete rooted trees for *tly* and *recA* are in supplementary figure S3, Supplementary Material online.

Table 1. Nucleotide Polymorphism in *recA* and *tly* Genes.

	n^a	L^b	L^{*b}	$L_S{}^b$	$L_{NS}{}^b$	R^c	Rm^c	Hd^d	Hn^d	S^e	s^e	$S_S{}^e$	$S_{NS}{}^e$	$\pi_S{}^f$	$\pi_{NS}{}^f$	$D_S{}^g$	$D_{NS}{}^g$
recA																	
P. Zappae	55	1,044	985	242	739	0.022	5	0.993	51	124	104	35	92	0.0071	0.0051	−2.561***	−2.843***
P. acnes	68	1,044	879	220	659	0.001	0	0.627	8	14	3	10	4	0.0124	0.0008	0.808	−0.757
tly																	
P. Zappae	15	696	291	78	213	0.000	0	0.467	4	13	12	78	213	0.0134	0.0038	−1.849*	−1.983*
P. acnes	43	696	696	184	509	0.000	0	0.808	12	34	13	184	509	0.0255	0.0068	0.412	−0.188

NOTE.—S, synonymous sites; NS, nonsynonymous sites.

[a]Number of sequences.

[b]Alignment length with (*L*) or without (*L**) sites containing gaps.

[c]Recombination rate (Hudson 1987) estimated between adjacent sites (*R*) and minimum number of recombination events (Rm).

[d]Haplotype diversity (Hd) and number of haplotypes (Hn).

[e]Number of segregating sites (*S*), of which *s* are singletons.

[f]Nucleotide polymorphism (Tajima 1983).

[g]Tajima's *D* (Tajima 1989).

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

contributing to most of the nucleotide variability (104 of the 124 single nucleotide polymorphisms are singletons). This observation is confirmed by the star like phylogeny (fig. 2*a*), the high haplotype diversity, and a strong skew in the site frequency spectrum of DNA polymorphism (as measured by Tajima's *D*; table 1). Interestingly, nonsynonymous polymorphism in *P.* Zappae's *recA* sites is more than 6-fold higher than in *P. acnes* orthologs extracted from the NCBI nucleotide database ($\pi = 0.0051$ vs. $\pi = 0.0008$, respectively; table 1). We can exclude a *P.* Zappae-specific large mutation rate because synonymous polymorphism of *recA* and polymorphism of *tly* and 16S are lower in *P.* Zappae than in *P. acnes* (possibly due to the bottleneck associated to the establishment of endophytism; table 1, in the 16S rDNA gene polymorphism was $\pi = 0.0$ and $\pi = 0.0016$ for *P.* Zappae and *P. acnes*, respectively). Rather, such polymorphic patterns suggest that the accumulation of nonsynonymous mutations is due to the absence (or strong relaxation) of purifying selection in *P.* Zappae's *recA*, as corroborated by four additional observations. First, three of the *P.* Zappae's *recA* sequences contain frameshift mutations that cause the appearance of premature stop codons (supplementary fig. S4, Supplementary Material online). Second, the mean rate of nonsynonymous substitutions (0.00244) is larger than the rate of synonymous substitutions (0.00239; Wilcoxon test, $P = 2 \times 10^{-8}$). Third, the ratio of nonsynonymous to synonymous polymorphism rates in *P.* Zappae, which can be used as a proxy for selective pressure (Schloissnig et al. 2013), is 0.124/0.145 = 0.86, very close to the neutral value of 1. Finally, the McDonald–Kreitman test revealed a strong excess of nonsynonymous polymorphisms ($P < 0.000001$). Interestingly, in *P.* Zappae, *recA* has also signatures of recombination, which are absent in *tly* as well as in *recA* of *P. acnes* (table 1).

Taken together, our results suggest a lack of selective constraints in *recA* of *P.* Zappae associated to loss of its function. In bacteria, loss of function may be beneficial whenever it entails a change, in the gene network, which turns out to be adaptive for specific metabolic needs (Koskiniemi et al. 2012; Hottes et al. 2013). We hypothesize that *recA* is not required for the endophytic life of *P.* Zappae, and that the resulting lack of functional constraints has favored the fast accumulation of nonsynonymous mutations. In fact, the degeneration of *recA* has rarely been observed in other bacteria, and it has been suggested that loss of *recA* function occurs during the initial stages of endocellular symbiosis (Dale et al. 2003). Our data therefore point towards the establishment of an obligate (probably endocellular) symbiosis of *P.* Zappae in grapevine. This conclusion finds reciprocal confirmation with the direct observation of *P.* Zappae by fluorescent microscopy (fig. 1*d*) and by our inability to cultivate any of the strains carrying a mutated *recA*. Efforts to cultivate *P. acnes* from the plant endosphere were in fact mostly unsuccessful, whereby only two *P. acnes* strains could be isolated on Nutrient Agar medium. These two strains had a slow evolving *recA* (in blue in fig. 2*c*), and their 16S sequences did not cluster with those typical of *P.* Zappae (in blue in fig. 2*a*). For this reason, we could not exclude that these isolates originated from environmental contaminations occurred after sampling and surface disinfection, and hence did not regard them as *P.* Zappae.

As *recA* is evolving neutrally, it can be safely used to estimate the age of *P.* Zappae endophytism in grapevine. Assuming a generation time of 5 h as reported for *P. acnes* (Hall et al. 1994), and assuming a mutation rate similar to that of the closely related *Mycobacterium tuberculosis* (2×10^{-10}, applied to synonymous sites; Ford et al. 2011), the age of *P.* Zappae can be set approximately 7,500 years ago, an age highly compatible with the domestication of grapevine. Date estimates are, however, known to be extremely dependent on the prior choice (e.g., Ometto et al. 2013; Rota-Stabelli et al. 2013); therefore, we have repeated our age estimates assuming varying generation times (to account for the fact that grape endosymbiotic *P.* Zappae may have a slow life cycle during cold winter season) as well as alternative mutation rates for bacteria (i.e., Drake 1991; Lee et al. 2012, see Materials and Methods for more details). Date estimates vary substantially, but in the majority of the cases they are compatible with the timescale of grapevine domestication

and cultivation (supplementary fig. S5, Supplementary Material online). As a further proof, we estimated the age of *P.* Zappae directly on the tree using Beast (Drummond et al. 2012) and a permissive mutation rate distribution, which set the origin of *P.* Zappae at approximately 6,300 years ago (supplementary fig. S6, Supplementary Material online). Our concordant age estimates reinforce the view that *P.* Zappae originated from human-associated *P. acnes*. We speculate that *P.* Zappae has exploited common agronomic practices, such as grafting and pruning, to transfer to the new host.

Our results represent the first evidence of human to plant horizontal transfer of an obligate symbiont and open new perspectives on the occurrence and significance of bacteria host transfer between humans and domesticated plants. Moreover, our findings underline how the extreme adaptability of bacteria and their ability to exploit and taking advantage of novel habitats can have unpredictable impact on host–pathogen evolution and eventually human health. The exploration of endophytic communities, using metagenome-based community analyses (Bulgarelli et al. 2012; Lundberg et al. 2012) will certainly allow precious insights in the occurrence and significance of bacterial endophytes. Likewise, the possible functional significance of the *P.* Zappae—grapevine interaction and the consequences for plant growth and health merits further investigation.

Materials and Methods

Plant Handling, DNA Isolation, and Polymerase Chain Reaction

Grapevine samples used for DNA extraction were obtained from field-grown and greenhouse cuttings in northern Italy. Plants were surface sterilized with ethanol and sodium hypochlorite as described before (Pancher et al. 2012). Twigs used for DNA extraction were decorticated with a scalpel to avoid contamination from superficial DNA in following reactions. Total DNA was isolated by freeze-crushing plant stems, followed by FastDNA spin kit for soil (MPBio, USA) extraction kit. The identity of bacterial colonies was confirmed by polymerase chain reaction (PCR) using standard 16S rDNA gene primers 16S-27F/16S-1492R (Lane 1991). Roche 454 GS FLX + system technology was used for pyrosequencing using primers 799f/1520R (Chelius and Triplett 2001). *Propionibacterium acnes*-specific *recA* and *tly* genes were PCR amplified using primer pairs PAR-1/PAR-2 and PAT-1/PAT-2, respectively (McDowell et al. 2005). Pyrosequencing of 16S rDNA amplicons was obtained using primers 799f/1520r with 454 adaptors and a sample-specific barcode. PCR was performed using High Fidelity FastStart DNA polymerase (Roche, USA) and 454 pyrosequencing was carried out on the GS FLX + system using the new XL + chemistry dedicated to long reads.

Evolutionary Analyses

Sequences of 16s rDNA, *recA* and *tly* from grapevine DNA extracts were aligned to all available homologs in GenBank (May 2013) (see the list in supplementary fig. S7, Supplementary Material online), and phylogenetic analysis performed by the maximum likelihood method in Phyml (Guindon et al. 2010) employing a nonparametric bootstrap procedure (100 pseudoreplicates) and the GTR + G model of nucleotide replacement. Population genetic analyses were done using DnaSp (Librado and Rozas 2009). Levels of nucleotide diversity were estimated using π (Tajima 1989), and recombination rate using the method proposed by Hudson (1987). Neutral equilibrium was tested by the Tajima's *D* test (Tajima 1989). To capture the evolutionary pattern following the endophytization, rates of nonsynonymous and synonymous substitutions were estimated only in each of the type IB sequences ($n = 51$; fig. 2) using the human a*sn1* sequence as outgroup. In addition, we estimated the (non)synonymous polymorphism rate as the ratio between the number of variable (non)synonymous sites in our sample and the total number of (non)synonymous sites. To test for the occurrence of positive or relaxed selection, we also used the McDonald–Kreitman test (Mcdonald and Kreitman 1991), which compares the polymorphism (p) and substitution (k) pattern in synonymous (S), and nonsynonymous (NS) sites: Because *P.* Zappae has no fixed substitution when using acnes as outgroup, we used *P. avidum* instead and obtained $p_S = 35$, $p_{NS} = 92$, $k_S = 112$, $k_{NS} = 6$.

FISH Microscopy

Plant tissues were harvested and frozen in liquid nitrogen and stored at −20 °C for long-term storage. Samples (0.5-mm long sections) were fixed overnight at 4 °C in a paraformaldehyde solution (4% in PBS), rinsed twice in PBS and dehydrated in an ethanol series (25%, 50%, 75%, and 99.9%; 15 min each step). A lysozyme 1 mg/ml^{-1} solution (in PBS) treatment was then applied to the samples for 10 min at 37 °C. FISH was carried out using probes bought at Genecust (Luxembourg). EUB mix (equivalent mixture of EUB338, EUB338II, EUB338III; Amann et al. 1990; Daims et al. 1999) coupled with a FLUOS fluorochrome, a HGC69a probe (for actinobacteria; Manz et al. 1992 coupled with Cy5, and a PAC 16S 598 probe (for *P. acnes*/*P.* Zappae; Poppert et al. 2010) coupled with a 405 dylight fluorochrome. NONEUB probe (Wallner et al. 1993) coupled with Cy5 was used independently as a negative control. Hybridization was also carried out with only PAC 16S 598 probe coupled with FLUOS. Hybridization was carried out at 46 °C for 2 h with 10–20 µl of solution (containing 20 mM Tris–HCl pH 8.0, 0.1% w/v SDS, 0.9 M NaCl, 25% formamide, and 10 ng/µl of each probe) applied on each plant samples placed on slides in a 50 ml moist chamber (with a piece of tissue inside imbibed with 5 ml of hybridization buffer). Posthybridization was carried out at 48 °C for 30 min with a post-FISH prewarmed solution containing 20 mM Tris–HCl pH 8.0, 0.1% w/v SDS, 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, and a NaCl concentration corresponding to formamide concentration. Samples were then rinsed with ice-cold distilled water before air drying for at least one day in the dark. The samples were covered with cover slips and mounted

with Citifluor AF1 (Citifluor Ltd., London, UK) before observation under confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G)laser FV10-LAHEG230-2). Pictures were taken at 405-, 488-, 633-nm wavelengths and under normal light and then merged (RGB) using the image J software. *Propionibacterium acnes*/*P.* Zappae appears as low blue/white fluorescent, actinobacteria (other than *P. acnes*) appear as low orange and other bacteria as green fluorescent. In case of use of the PAC 16S 598 probe coupled with FLUOS, *P. acnes*/*P.* Zappae appeared as green fluorescent.

Estimation of *P.* Zappae Age

Although estimating the age of *P.* Zappae from *recA* we used a mutation rate μ ranging from 1×10^{-10} to 6×10^{-10} mutations/bp/generation, which encompasses the value of 2.1×10^{-10} estimated for *M. tuberculosis* (Ford et al. 2011), the closest bacterium for which a mutation rate has been estimated, as well alternative bacteria mutation rates (i.e., the values of $\sim 6 \times 10^{-10}$ and $\sim 2 \times 10^{-10}$ of *E. coli* reported by Drake [1991] and Lee et al. [2012], respectively). We based our calculations on the 35 variable synonymous sites (total sites $L_S = 242$; table 1) identified among the 55 *P.* Zappae *recA* clones (adding nonsynonymous sites did not substantially change the estimates, results not shown). We assumed a generation time G_T ranging from 1 to 24 h, thus including the estimate of 5 h reported for *P. acnes* in culture (Hall et al. 1994). It is however questionable whether *P.* Zappae has the same generation time of human *P. acnes*, as daily and seasonal temperature variation (e.g., cold winters) suggest fewer generations per year, therefore a longer average generation time than the human counterpart. We could then estimate the age in years, T_Z, of *P.* Zappae as $T_Z = (35/55)/(\mu \times L_S \times h/G_T)$, where $h = 8{,}765.81$ is the number of hours in a year (after correcting for leap years). Using the above estimates, T_Z ranged from a minimum of 500 years ($\mu = 6 \times 10^{-10}$ mutations/bp/generation and $G_T = 1$ h) to a maximum of 71,996 years ($\mu = 1 \times 10^{-10}$ mutations/bp/generation and $G_T = 24$ h), with a core of estimates of ~3,000–7,000 years, corresponding to the most likely mutation rates and generation times (supplementary fig. S5, Supplementary Material online).

We further estimated age of *P.* Zappae employing a molecular clock technique on the 16S phylogeny. We used Beast (Drummond et al. 2012) assuming no priors at neither roots or any of the nodes and employing a log-normal clock model with a normally distributed mutation rate of mean 0.568 (according to Drake 1991) and a permissive standard deviation of 0.29 to allow sampling most of the mutation rates explored in supplementary figure S5, Supplementary Material online.

Supplementary Material

Supplementary figures S1–S7 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

A.C. conceived the study. A.C., O.R.-S., and L.O. designed the research. S.C. performed FISH. M.P., A.C., and L.A. collected samples and generated and assembled gene data. O.R.-S. performed phylogenetic analyses. L.O. performed population genetic analyses. I.P., G.A., C.V., S.C., and A.S. contributed reagents and improved the manuscript. A.C. drafted the manuscript with O.R.-S. and L.O. This work was funded by Provincia Autonoma di Trento, progetto PAT – Call 2 Team 2009 – Incoming – Mecagrafic to A.C., and progetto PAT Call 3 COFOUND 2010 to O.R.S. The authors thank COST Action FA1103 for supporting this work.

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