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Propionibacterium acnes populations involved in deep pathological samples and their dynamics along the cardiac surgical pathway

S. Romano-Bertrand · M. Beretta · H. Jean-Pierre ·
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Abstract *Propionibacterium acnes* belongs to the normal skin microbiota, but it is also responsible for acne vulgaris and causes serious infections such as endocarditis and surgical site infections (SSI). The *P. acnes* population is structured into phylogenetic groups, with phylotype I being associated with acne. Herein, we explore the link between phylotypes and clinical origins in a collection of *P. acnes* isolated from different body sites, involved in deep infections or healthcare-associated infections (HAI), with particular emphasis on strains from cardiac SSI. Cardiac SSI have been further studied in terms of *P. acnes* population dynamics during the care pathway. The *recA* and *tly* genes phylotypes were compared to hemolytic behavior, susceptibility to antimicrobial agents, and clinical origins. An original approach of *recA* polymerase chain reaction temporal temperature gel electrophoresis (PCR-TTGE) was developed and applied for the direct identification of *P. acnes* phylotypes in surgical samples, in order

to assess their temporal dynamics during the surgical course. Our results underlined the preferential involvement of IA-2/IB and II phylogroups in HAI and SSI. Unlike IA and II, type IA-2/IB presented a gradual increase with the depth of sampling in the peroperative phase of cardiac surgery. Phylotypes IA and IA-2/IB were both predominant in scar tissues and on postoperative skin, suggesting a specific predisposition to recolonize skin. Particular association of the phylotype IA-2/IB with SSI and its propensity to colonize wounds in cardiac surgery was observed. We assumed that the follow-up of *P. acnes* phylotypes during pathological processes could give new clues for *P. acnes* pathogenicity.

Introduction

Propionibacterium acnes is an anaerobic Gram-positive bacillus member of the human microbiota mainly on skin [1], but also in the oral cavity, and digestive and genital tracts [2]. In clinical samples, *P. acnes* is often overlooked as a contaminant and is underestimated due to its growth requirements [3–5]. However, *P. acnes* can produce putative virulence factors involved in inflammation processes [4], as observed in acne vulgaris [5] and other inflammatory pathologies, such as sarcoidosis [6], or potentially in prostatic pathologies [7]. *P. acnes* can also cause severe deep infections like endocarditis [8–10] and surgical site infections (SSI) in orthopedic [11, 12], cardiac [13, 14], or neurological surgeries [15].

Metagenomics [16] and comparative genomics [17] showed strain variations supporting the existence of specific *P. acnes* subpopulations. The strain HL096PA1 causing severe acne displayed a plasmid encoding adhesion factors and a high amount of pseudogenes, suggesting its adaptation to pathogenic behavior in a narrow niche [18]. Moreover, population genetics indicated the roles of distinct lineages of *P. acnes* in different diseases. In this context, several

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multilocus sequence typing (MLST) schemes, including from 3 to 9 loci according to studies, were proposed. They identified three main distinct phylotypes, named I, II, and III [19–23]. Phylotype I is subdivided into groups IA (subdivided into IA-1 and IA-2), IB, and IC [23, 24], but recombination events occur between IA-2 and IB groups, leading to misidentification of these subtypes [25]. The genes *recA* (recombinase A) and *thy* (putative hemolysin) were widely used in MLST schemes because they presented satisfactory discriminative power among the three phylotypes I, II, and III [23, 26]. The association of *recA* and *thy* allowed the identification of IA-1 but not the separation of IA-2 from IB [25]. Moreover, *recA* structuration is correlated to antigens, cell wall sugar, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) types [27].

Phylotype I appeared to be the most prevalent in the whole population of *P. acnes* and gathered mainly isolates from acne and healthy skin [23, 24]. Division IA comprised the majority of isolates from inflammatory acne lesions [28]. It included the epidemic clone ST18 (=eST1) and its descendants in the IA-1 group [22, 25], which were never detected in healthy skin [20].

Besides phylotype IA and acne, links between genetic group and pathogenic behavior were not fully meaningful. Isolates of phylotypes II and III were described as originating mainly from healthcare-associated infections (HAI) [19], but this association was not exclusive because type I strains were also involved in HAI [20]. Types IB and II were often encountered in prostatectomy specimens from cancer [29], without prediction of infection or disease [30]. Similarly, phylotypes IB and II were often detected during revising prosthetic surgeries, but their presence did not differentiate infection from colonization [11]. Despite an unclear association between a particular genetic group and a specific pathogenic behavior in some cases, the published data lend support to the view that pathogenic versus truly commensal lineages of *P. acnes* may exist [25]. The determination of relationships between lineages and infections is likely to have important therapeutic and diagnostic implications [25].

In cardiac surgery, *P. acnes* is the third most prevalent bacterium in deep SSI [13, 31, 32], making it important to consider. A previous study based on universal 16S polymerase chain reaction temporal temperature gel electrophoresis (PCR-TTGE) describing the dynamic of bacterial communities in cardiac surgery wounds showed high rates of *Propionibacterium* spp. at different stages of patients' hospitalization [31]. However, no study has yet described the phylotypes involved in cardiac SSI, preventing rare isolates from SSI being included in the currently available MLST studies [19, 25].

The purpose of this study was to explore the links between the main phylotypes based on *recA* and *thy* sequencing and clinical origins, hemolytic behavior, and resistance in a collection of *P. acnes* involved in deep infections or HAI, with particular emphasis on strains from cardiac SSI. Based on the

recA phylogeny, an original culture-independent method was applied for the direct identification of *P. acnes* phylotypes involved in cardiac surgical samples in order to assess their dynamics during the surgical pathway of the patients.

Materials and methods

Propionibacterium acnes isolates, culture conditions, antibiotic susceptibilities, and hemolysis test

Ninety-nine *P. acnes* clinical isolates collected from different infectious sites between May 2011 and March 2013 at the Hospital of Montpellier (France) were analyzed (Table 1). The isolates were included considering at least one of the following criteria: (i) isolation in normally sterile sites, (ii) isolation in deep infectious processes, (iii) antimicrobial assay on the isolate for therapeutic purposes, (iv) prescription of antimicrobials targeted against *P. acnes*. Among the 99 strains, 27 were isolated from cardiothoracic samples (including 19 from cardiac SSI). Hemolysis tests were performed by culture on nutritive medium supplemented with 5 % sheep blood (COS Agar, bioMérieux, France) at 37 °C in an anaerobic atmosphere. The degree of hemolysis was assessed after 72 h of culture and noted negative if absent, weak if present but light or moderate, and strong if important or total. Antibiotic susceptibilities were tested for cefamandole, vancomycin, and clindamycin by an agar diffusion assay (members of the Société Française de Microbiologie committee, 2003).

Surgical samples and ethic statements

Surgical site samples were obtained from 14 patients undergoing cardiac surgery at the Cardio-thoracic Surgery Unit of Montpellier University Hospital (France). They were collected superficially and deeply at the different steps of hospitalization (before hospitalization, during the surgical procedure, during postoperative hospitalization, and 3 months after discharge). Primary human materials used in this study were collected on sterile cotton swabs as performed for the routine clinical diagnostic process without change in the surgical procedures or nursing care. Each patient included gave oral informed consent. The study proposal was approved by the ethical committee of our institution: Comité de Protection des Personnes Sud Méditerranée IV, N°ID - RCB: 2011-A00078-33.

DNA extraction and PCR amplification

DNA was extracted from colonies or from surgical samples on cotton swabs by an enzymatic method (MasterPure Gram Positive DNA Purification Kit, Epicentre, France) and diluted at a final concentration of 50 ng/μL. The 16S rRNA gene was amplified using the primers HDA1f–HDA2r [31, 33]. The

Table 1 Origins, genotype, and hemolytic capacity of the *Propionibacterium acnes* isolates. ^aAccording to the nomenclature of the <http://pacnes.mlst.net/> database. ^bAccording to the nomenclature of the <http://pubmlst.org/pacnes/> database. Allele numbers marked with an

asterisk correspond to new alleles displaying >99 % identity in the sequence with the original allele. *SSI* surgical site infections, *STM* skin-tissue-muscle. *IB* corresponded to IA-2/IB, the two types being undistinguishable by *recA/tly* typing

Strains and origins	<i>recA</i> type	<i>tly</i> type	<i>recA</i> allele ^a (753 pb)	<i>recA</i> allele ^b (463 pb)	<i>tly</i> allele ^b (777 pb)	Hemolysis		
Cardiothoracic isolates (<i>n</i> =27, including cardiac SSI isolates, <i>n</i> =19)	PA15	Purulent effusion from sternal wound	II	II	6	2	10	–
	PA23	Sternal wound	IB	IB	2	1	8	++
	PA38	Sternal wound	IA	IA	5	1	1	+++
	PA54	Heart defibrillator	IB	IB	2	1	8	++
	PA74	Pacemaker lodge	IA	IA	5	1	1	–
	PA75	Left ventricular pacemaker sensor	IA	IA	5	1	1	–
	PA76	Postoperative mediastinal hematoma	IB	IB	2	1	8	–
	PA76b		IB	IB	2	1	8	–
	PA83	Aortic valve	IB	IB	2	1	8	–
	PA83b		IB	IB	2	1	8	–
	PA83t		IB	IB	2	1	8	–
	PA86	Sternal wound	IA	IA	5	1	1	+
	PA92	Sternum	IA	IA	5	1	1	+++
	PA93	Sternum	IA	IA	5	1	1	++++
	PA94	Sternal wound	IB	IB	2	1	8	++
	PA95	Purulent effusion from sternal wound	IB	IB	2	1	8	++
	PA98	Valve	II	II	6	2	10	–
	PA99	Pleural injury	IB	IB	2	1	8	+++
	PA100	Tricuspid valve	II	II	6	2	10	–
	PA103	Pacemaker	IA	IA	5	1	1	–
	PA104	Aortic ring	IB	IB	2	1	8	–
	PA105	Pacemaker lodge	IA	IA	5	1	1	++++
	PA109	Sternum	IB	IB	2	1	8	+++
	PA110	Pacemaker effusion	IA	IA	5	1	1	–
	PA113	Pacemaker lodge	IB	IB	2	1	8	+++
	PA114	Sternal wound	IA	IA	5	1	1	++++
	PA115	Sternal wound effusion	IB	IB	2	1	8	++
Postoperative orthopedic isolates (<i>n</i> =14)	PA14	Shoulder bladder	IB	IB	2	1	8	+
	PA19	Knee prosthesis	IB	IB	2	1	8	+
	PA28	Femoral biopsy	IA	IA	5	1	1	++
	PA31	Femoral shaft	IA	IA	5	1	1	–
	PA31b	Osteoarticular capsule	II	II	6	2	10	–
	PA64	Sacrum bone	IB	IB	2	1	8	++
	PA70	Superficial wound infection (osteoarticular)	IB	IB	2	1	8	++
	PA72	Knee synovial fluid	IB	IB	2	1	8	+++
	PA73	Hip hematoma	IB	IB	2	1	8	+++
	PA85	Spondylitis	II	II	6	2	10	–
	PA87	Left humerus	III	III	9	4	12	–
	PA88	Left humerus prosthesis	IB	IB	2	1	8	+
	PA89	Left humerus prosthesis	IB	IB	2	1	8	++
PA90	Left humerus prosthesis	II	IB	8*	2	8	–	
Oropharyngeal isolates (<i>n</i> =12)	PA6	Left maxillary sinus	IA	IA	5	1	2	–
	PA17	Outer ear	IA	IA	5	1	1	+++
	PA18	Outer ear	IB	IB	2	1	8	+++
	PA45	Infected skin of skull	IA	IA	5	1	1	++++

Table 1 (continued)

Strains and origins			<i>recA</i> type	<i>tly</i> type	<i>recA</i> allele ^a (753 pb)	<i>recA</i> allele ^b (463 pb)	<i>tly</i> allele ^b (777 pb)	Hemolysis
	PA55	Cervical lymphadenopathy	IA	IA	5	1	1	–
	PA61	Cervical ganglion	II	II	6	2	10	–
	PA68	Maxillary sinus	IB	IB	2	1	8	+++
	PA78	Left cheek cyst	IA	IA	5	1	1	+++
	PA91	Submandibular mass	II	II	8	2	10	–
	PA91b		II	II	8	2	10	–
	PA96	Ear paracentesis	II	II	6	2	10	–
	PA107	Postoperative cervical abscess	IA	IA	5	1	1	++
Neurosurgical isolates (<i>n</i> =11, including neuro-SSI isolates, <i>n</i> =5)	PA25	Cerebrospinal fluid	IA	IA	5*	1*	1	–
	PA27	Cerebral biopsy	IB	IA	2	1	1	+++
	PA41	Cranial bone	IA	IA	5	1	1	+++
	PA43	Cerebrospinal fluid	IB	IB	2	1	8	–
	PA48	Cerebrospinal fluid	III	III	9	4	12	–
	PA50	Cerebrospinal fluid	IA	IA	5	1	1	+++
	PA52	Cerebrospinal sample	IA	IA	5	1	1	++
	PA101	Craniotomy scare	IA	IA	5	1	2	–
	PA102	Subdural fluid	IA	IA	5	1	2	–
	PA106	Postneurosurgical subcutaneous hematoma	IA	IA	5	1	1	++++
	PA108	Postoperative superficial cranial wound	II	II	6*	2	10	–
Neonatal isolates (<i>n</i> =8)	PA5	Gastric liquid of newborn	IA	IA	5	1	1	–
	PA12	Gastric liquid of newborn	IA	IA	5	1	1	–
	PA13	Gastric liquid of newborn	IB	IB	2	1	8	++
	PA58	Newborn liver	IB	IB	2	1	8	–
	PA67	Gastric liquid of newborn	IB	IB	2	1	8	++
	PA69	Gastric liquid of newborn	IB	IB	2*	1	8	++++
	PA82	Gastric liquid of newborn	IB	IB	2	1	8	+++
	PA111	Gastric liquid of newborn	IA	IA	5	1	1	++++
Ophthalmologic isolates (<i>n</i> =6)	PA29	Corneal abscess	IA	IA	5	1	1	+++
	PA46	Left sinus	IA	IA	5	1	1	+
	PA57	Left eye	II	II	6*	2*	10	–
	PA77	Right eye	IA	IA	5	1	1	+
	PA80	Conjunctival secretions	IB	IB	2	1	8*	+
	PA81	Conjunctival secretions	IA	IA	5	1	1	+++
Abdominal isolates (<i>n</i> =6)	PA36	Ascites	II	II	6*	2*	10	–
	PA39	Oviduct	IB	IB	2	1	8	+++
	PA40	Parietal cyst	IB	IB	2	1	8	+
	PA42	Semen culture	IA	IA	5	1	13	++
	PA47	Peritoneal fluid	III	III	9	4	12	–
	PA49	Peritoneal dialysis fluid	IA	III	5	1	13	–
STM isolates (<i>n</i> =5)	PA7	Muscle biopsy	IB	IB	2	1	8	+
	PA44	Calf wound	IB	IB	2	1	8	+
	PA60	Back wound	IA	IA	5	1	1	+++
	PA62	Ganglion	II	II	6	2	10	–
	PA79	Necrotic wound	IA	IA	5	1	1	–
Blood samples (<i>n</i> =3)	PA9	Blood culture	IB	IB	2	1	8	+++
	PA53	Blood culture	IB	IB	2	1	8	+++
	PA65	Blood culture	II	II	6	2	10	+++
Unknown origin (<i>n</i> =7)	PA22	Unknown	II	II	6	2	10	+

Table 1 (continued)

Strains and origins			<i>recA</i> type	<i>tly</i> type	<i>recA</i> allele ^a (753 pb)	<i>recA</i> allele ^b (463 pb)	<i>tly</i> allele ^b (777 pb)	Hemolysis
PA32	Unknown		IB	IB	2	1	8	++
PA33	Unknown		IB	IB	2	1	8	++
PA34	Unknown		IB	IB	2	1	8	+++
PA51	Unknown		II	II	6	2	10	–
PA56	Unknown		II	II	6	2	10	–
PA84	Unknown		II	II	8	2	10	–

recA and *tly* genes were amplified by the primers PAR-1 (5'-AGCTCGGTGGGGTTCTCTCATC-3') and PAR-2 (5'-GCTTCCTCATACCACTGGTCATC-3') and PAT-1 (5'-CAGGACGTGATGGCAATGCGA-3') and PAT-2 (5'-TCGTTCAACAAGACCACAGTAGC-3'), respectively [24].

A nested PCR approach was needed because of the low bacterial load in surgical samples (<10³ UFC/tube, assessed by 16S rRNA gene-based PCR as previously described [31]). The *recA* nested PCR approach consisted of a pre-amplification (with primers PAR-1 and PAR-2), followed by a second amplification of a 333-pb fragment overlapping the terminal region of the *recA* gene, using the primers PR264-GC (corresponding to the primer PR264 5'-GCAGGCAGAGTTTGACATCC-3' [30] with a "GC clamp" rich in GC added at the 5'- extremity) and PAR-2. The PCR mixture of 50 µL consisted of 1 µL of DNA extract, 200nM of each primer, 200 mM of each dNTP (Fermentas), and 2.5U FastStart Taq DNA Polymerase (Roche, France) in the appropriate reaction buffer containing 1.8 mM MgCl₂. PCR conditions were an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 64 °C for 30 s, and 72 °C for 1 min 30 s, and a final elongation at 72 °C for 10 min.

Sequencing and phylogenetic analyses on *recA* and *tly* genes

Amplification products were sequenced using both primers PAR-1 and PAR-2 for *recA* and forward primer PAT-1 for *tly* on an ABI 3730xl sequencer (Cogenics, France). Sequences were deposited in the GenBank database (accession numbers KJ572580 to KJ572777). Gene sequences were aligned using ClustalW [5] in order to determine the polymorphic positions. A number was attached to each sequence according to the *recA* and *tly* alleles available in the <http://pacnes.mlst.net/> and <http://pubmlst.org/pacnes/> databases. Phylogenetic trees were constructed based on maximum likelihood (ML) analysis using PhyML software (<http://www.phylogeny.fr>). The general time-reversible (GTR) model plus gamma distribution and invariant sites was used as the substitution model and bootstrap supports were computed after 100 reiterations. Reference *recA* sequences for the types IA (AY642073), IB (AY642092), II (AY642090), and III (DQ672252) were

included in the phylogenetic analysis [21, 26]. An isolate that belonged to a clade containing a reference sequence was affiliated to the corresponding phylotype. Some *tly* and *recA* alleles in the subphylotype IA-2 are shared with phylotype IB [25]. Therefore, the phylotype IB will be named IA-2/IB in this study to avoid confusion.

recA-based phylotyping by temporal temperature gel electrophoresis

TTGE migration was performed in the DCode Universal Mutation Detection System (Bio-Rad Laboratories). Gels contained 7 % (w/v) bisacrylamide (37.5:1), 7 M urea, 40 ml N,N,N₉,N₉-tetramethylethylenediamine, and 0.1 % (w/v) ammonium persulfate, and were run in 16 Tris acetate EDTA (TAE) buffer at pH 8.3. The electrophoresis migration conditions were a pre-migration at 20 V for 15 min and a migration of 17.5 h at 53 V from an initial temperature of 67 °C to a final temperature of 70.5 °C (increase of 0.2 °C per hour). Bands were visualized on UV illuminator after gel incubation in an ethidium bromide bath. For band sequencing, gel slices excised with a sterile scalpel were washed twice in DNA-free water and DNA was eluted by an overnight incubation in 10 mM Tris buffer (pH 8.5) at 37 °C. Amplification of the 5'-terminal *recA* region was performed using 1 µL of band eluate and the primers PR264 without GC clamp and PAR-2 as previously described. PCR products were sequenced on an ABI 3730xl sequencer (Beckman Coulter Genomics) and compared to reference *recA* sequences for phylotype affiliation.

Results

Phylogenetic structure of the *P. acnes* clinical collection based on *recA* and *tly* genes

The *recA* and *tly* consensus sequences of 1,119 bp and 848 bp, respectively, were obtained for the 99 clinical isolates. Partial sequences were compared and numbered according to the MLST databases (Table 1). The alignment of the 1,119-bp

recA sequences showed 22 polymorphic sites (1.97 % against 1.86 % for the 753-pb *recA* sequence used in the MLST databases), whereas the 848-bp *tly* sequences were more variable, with 39 polymorphic positions (4.6 % against 4.12 % for the 777-pb *tly* sequence used in the MLST databases). Phylogeny affiliated *recA* clades to phylotypes according to the reference sequences included in the analysis (Fig. 1). Tree topologies appeared robust and were globally congruent between *recA* and *tly* markers (Fig. 1), except for strains PA27, PA49, and PA90 (corresponding, respectively, to phylotypes IA-2/IB, IA and II in the *recA* tree, and to phylotypes IA, III, and IA-2/IB in the *tly* tree). These incongruences suggested recombination among phylotypes. It was noteworthy that the group PA6/PA101/PA102 was in phylotype IA in the *recA* tree and in a yet undescribed phylotype with a new allele related to allele 2 in the *tly* tree. The phylogenetic structure and isolates distribution did not significantly change when the concatenated sequence were analyzed. However, the *tly* undescribed clade (PA6, PA101, and PA102) was supported by a high bootstrap value in the concatenated tree, suggesting the emergence of a robust subpopulation (data not shown).

The clades IA and IA-2/IB gathered the majority of isolates (77/99) in both the *recA* and *tly* trees. However, some atypical

isolates in each clade differed from the reference sequences and from the majority of isolates. Among them, PA25 and PA69 displayed undescribed new *recA* alleles. PA99, PA109, and PA73 had allelic affiliation in MLST, but their incongruent phylogenetic positions were due to mutations out of the region used for MLST, suggesting the emergence of new lineages in phylotype I by gene mutation rather than by gene exchange.

Phylotype II was less robust and displayed higher diversity in the *recA* tree. However, the majority of isolates ($n=12$) were grouped with the reference sequences and corresponded to the *recA* allele 6 in the MLST scheme in <http://pacnes.mlst.net/> (Fig. 1, left side). The other isolates ($n=7$) were distributed in six branches supported by low bootstrap values, making their relative phylogenetic positions uncertain. They corresponded to alleles 6 and 8, but were also related to new alleles in <http://pacnes.mlst.net/>. In the *tly* tree, phylotype II displayed low diversity and formed a robust clade (Fig. 1, right side) that fully matched *tly* allele 10 in MLST. Finally, phylotype III was the least represented in our collection ($n=3$ in the *recA* tree and $n=4$ in the *tly* tree), which displayed low variability and high robustness.

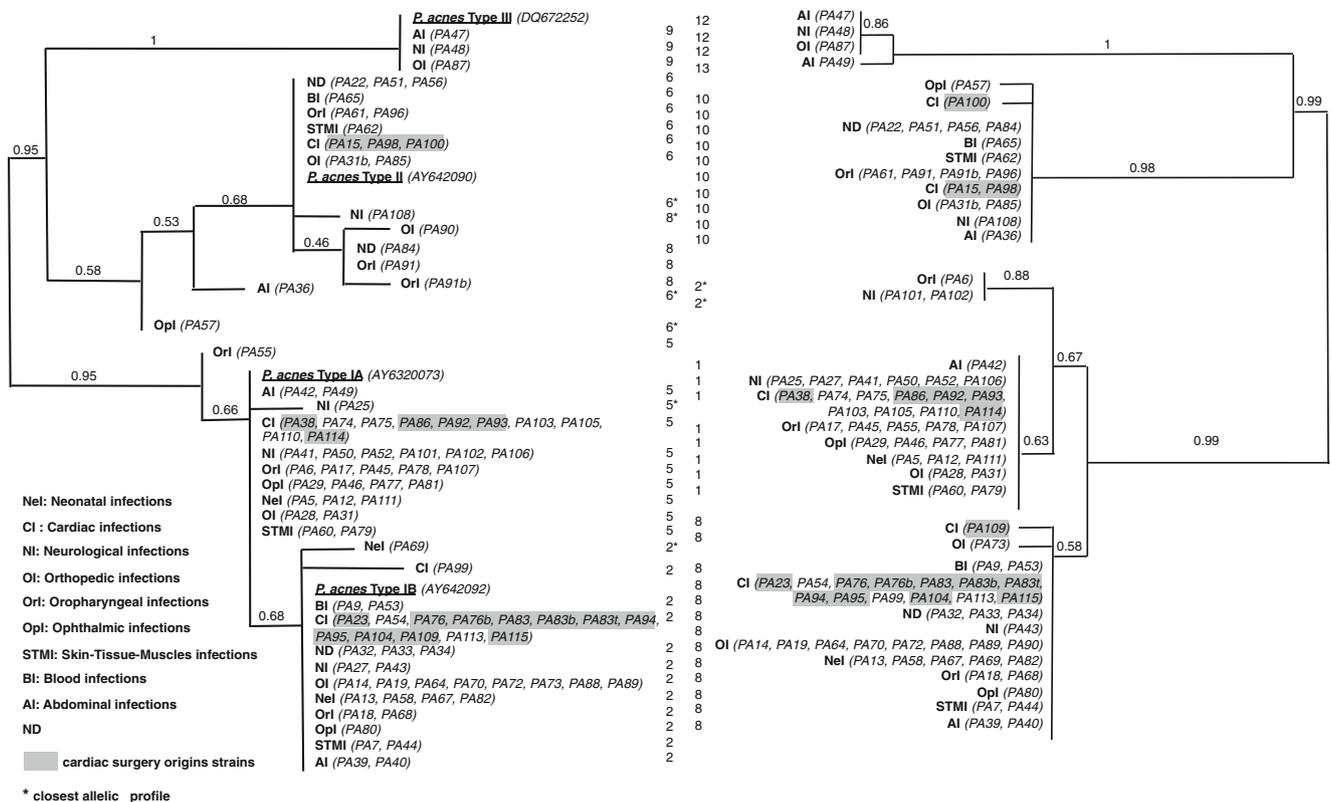


Fig. 1 Multilocus (ML) phylogenetic trees reconstructed from *recA* and *tly* sequences of *Propionibacterium acnes* isolates, left and right sides, respectively. The *recA* sequences of reference strains (underlined) are included for phylotype affiliation. For each branch, the corresponding allele is numbered according to the MLST scheme deposited in [http://](http://pubmlst.org/pacnes/)

pubmlst.org/pacnes/. Allele numbers marked with an asterisk correspond to new alleles displaying >99 % identity in the sequence with the most related described allele. The numbers at the nodes are bootstrap values. Isolate names are in parentheses beside their clinical origin. Isolates from cardiac surgical site infections (SSI) origin are shaded

Links between phylotypes and clinical origin

Phylotype I was largely the most prevalent in our collection focused on strains involved in deep or severe infections (from 67 to 100 %, depending on the clinical origin). Type II represented 19.2 % of the collection and type III was represented by only three strains (Fig. 2). The distribution of clinical isolates by phylotypes appeared to be globally independent of their origin (Fig. 2). However, some particularities deserved to be underlined. Strains from the abdominal area and from orthopedic or neurological infections belonged to the four phylotypes IA, IA-2/IB, II, and III, and phylotype III was not detected from the other origins. On the contrary, the eight isolates from neonates, mostly from gastric liquid, belonged to type I. Phylotype IA was mainly found in head and neck infections, with 68 %, 63 %, and 45 % from ophthalmologic, neurological, and oropharyngeal origins, respectively. For all the other clinical origins, the genetic diversity of isolates was dominated by phylotype IA-2/IB. This is noteworthy for strains implicated in orthopedic surgery and bacteremia. Among the oropharyngeal strains included herein, type II was particularly prevalent (36.4 %). The distribution in phylotypes of the 27 isolates from cardiothoracic infections, also considering the strains from cardiac SSI separately, was roughly similar to that of the whole population (Fig. 2).

Hemolytic phenotypes in clinical strains

The repartition in phylotypes of the hemolytic behavior for the 99 isolates is presented in Fig. 3a. Non-hemolytic strains were distributed in the *recA*-*tly* phylotypes IA, IA-2/IB, II, and III. No strains in phylotype III and only 2 isolates among 18 in

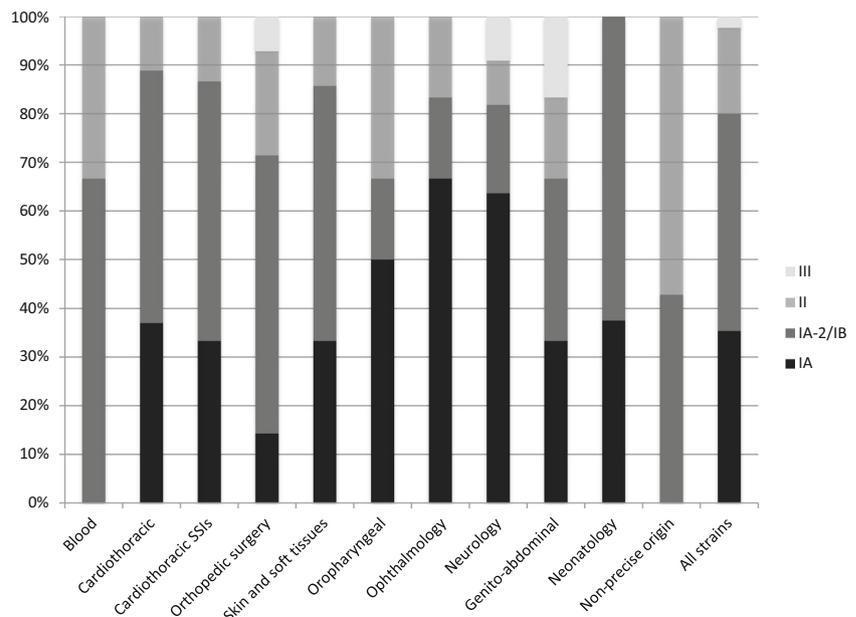
phylotype II were hemolytic. In contrast, phylotype I was weakly (33.7 %) or strongly hemolytic (36.3 %). Phylotypes IA and IA-2/IB displayed 61 % and 78 % hemolytic strains, respectively. Of note, strain PA49 corresponding to *recA* type IA and *tly* type III was non-hemolytic, as for other typical type III strains.

The hemolytic capacities appeared to be rather related to the phylotype but varied slightly according to the origin of infections (Fig. 3b, c, d). The distribution of cardiothoracic isolates was roughly similar to that of the whole population, except for a few strains, mainly of type IA-2/IB, which were more often non-hemolytic in cardiothoracic isolates (Fig. 3b). Of note, the six isolates from cardiac valves were non-hemolytic IA-2/IB or II types, whereas strains from sternal wounds were mostly hemolytic (10/11) (Table 1). Non-hemolytic IA strains were all isolated from pacemaker lodge or devices. Cardiothoracic and neurological IA strains were either non-hemolytic or strongly hemolytic (Fig. 3b, d). No obvious hemolytic phenotype was related to neurological infections. In orthopedics, phylotype IA-2/IB dominated and was always hemolytic but often weakly (Fig. 3c). The three strains from blood culture were strongly hemolytic.

Identification of the main phylotypes by *recA* PCR-TTGE

The denaturing gel PCR assay was based on the genetic diversity of the 3' part of the *recA* gene, containing 13 polymorphic sites in a 333-bp fragment (Table 2). This method was applicable to complex microbiota without subculturing and allowed the direct detection of the main *recA* phylotypes in a single experiment, by comparison to a ladder (Fig. 4).

Fig. 2 Relative prevalence of the different phylotypes of *P. acnes* according to clinical origin



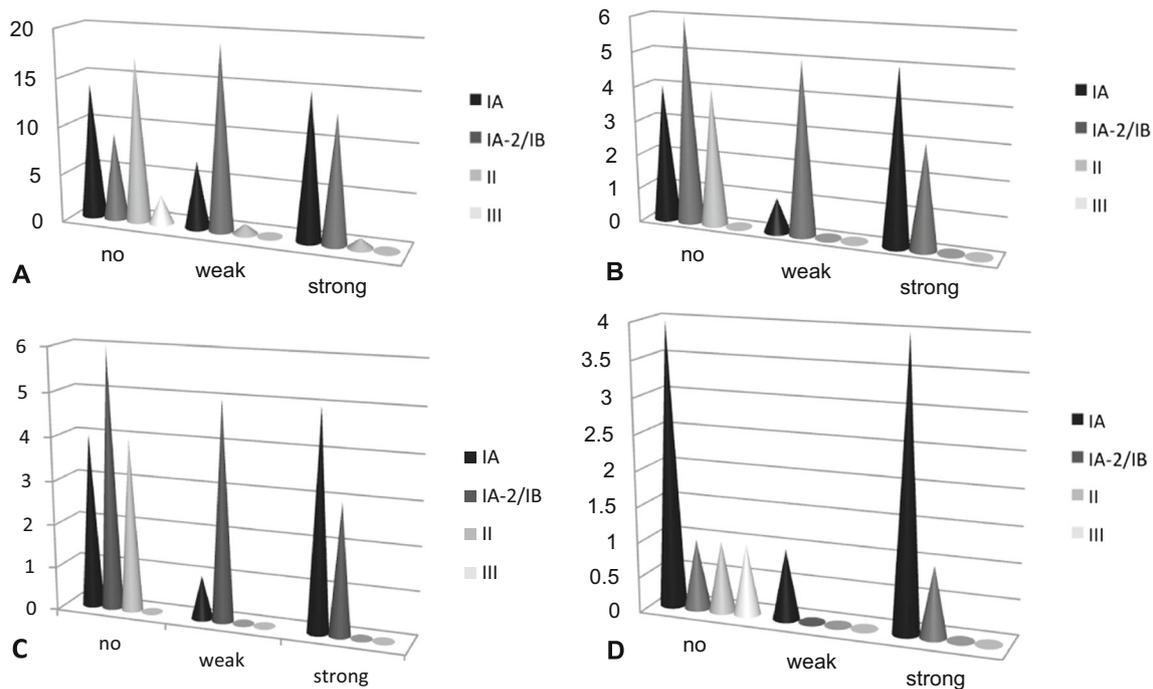


Fig. 3 Hemolytic phenotype according to the phylotype for: (a) the whole *P. acnes* population, (b) isolates from cardiothoracic infections, (c) isolates from orthopedic infections, and (d) isolates from neurological infections

Single nucleotide polymorphisms (SNP) in the 3' part of the *recA* gene according to the types of sequence and the isolates are given in Table 2. Thirty-five of the 36 isolates belonging to phylotype IA shared an identical sequence type (IA-3'*recA*1). The strain PA55, remote from the other IA strains in the *recA* tree, displayed a T/C mutation in position 275 (IA-3'*recA*2), which conferred to the corresponding 333-bp fragment a particular migration behavior in TTGE (Fig. 4). Almost all (41/42) of the type IA-2/IB strains shared the same allele (IA-2/IB-3'*recA*1). PA69 isolated from a neonatal gastric fluid was the sole strain with IA-2/IB-3'*recA*2 because of an SNP (C/A) in position 125. Despite a single SNP (G/A) in position 167 between IA-3'*recA*1 and IA-2/IB-3'*recA*1, they were clearly separated in TTGE (Fig. 4). The three clinical isolates PA47, 48, and 87 belonging to type III in the *recA* tree shared the same sequence type III-3'*recA*1 that migrated differentially from other ST. The type III-3'*recA*1 TTGE band displayed a fuzzy aspect compared to bands corresponding to the other phylotypes (Fig. 4).

As expected from the *recA* tree topology, phylotype II was more variable, with four different ST that roughly corresponded to the four branches in the tree (Fig. 1). Allele II-3'*recA*1 was shared by 13 of the 19 type II strains (Fig. 4), mostly grouped in the main cluster that contains the reference sequence of phylotype II in the *recA* tree (Fig. 1). Four clinical isolates presented the same II-3'*recA*2, differing from II-3'*recA*1 by one SNP (C/T in 98). The three other type II clinical strains PA36, 37, and 57 in the external position in the *recA* tree (Fig. 1) displayed two different sequences (Fig. 4).

Finally, phylotype II displayed four migration profiles, each differing from the other phylotypes. The ten bands corresponding to ten 3'-*recA* types were included in a ladder to represent the *recA* PCR-TTGE diversity of our collection (Fig. 4).

P. acnes phylotypes involved in cardiac surgery

We obtained 154 superficial and deep samples from 14 patients during the different stages of hospitalization in cardiac surgery. Seventy samples were positive when they were screened for significant bacterial load using universal 16S rRNA gene PCR. Therefore, these samples constituted the set for the direct detection of the *P. acnes* phylotypes by *recA* PCR-TTGE. Among them, 54 were collected during the surgical procedure, 34 superficially (19/34 and 15/34 cutaneous and subcutaneous samples, respectively) and 20 deeply (11/20 and 9/20 sternal edges and mediastinum samples, respectively). Seven samples concerned the skin before hospitalization, seven samples the skin and scar tissues after the surgical intervention, and only two samples involved the skin 3 months after the surgery. The TTGE fingerprints of ten representative samples are shown in Fig. 4. The phylotypes present in the 70 perioperative samples were identified by comparison to the diversity ladder. Four bands that did not match the ladder were separately analyzed and did not correspond to any known sequence type (data not shown).

The *P. acnes* phylotyping for all perioperative and peroperative samples from each patient is presented in Table 3.

Table 2 Phylotypes of the 3'-end of the *recA* gene sequence types (ST) of *P. acnes* isolates with the corresponding polymorphic sites. *The nomenclature indicates the phylotype followed by the sequence type of the 3' part of the *recA* gene

Phylotypes*	Clinical isolates	Nucleotidic polymorphic sites												
		1	2	3	4	5	6	7	8	9	10	11	12	13
IA-3'recA1	PA5, 6, 12, 17, 25, 28, 29, 31, 38, 41, 42, 45, 46, 49, 50, 52, 60, 74, 75, 77, 78, 79, 81, 86, 92, 93, 101, 102, 103, 105, 106, 107, 110, 111, 114	T	C	C	C	C	G	G	T	G	T	C	A	T
IA-3'recA2	PA5bis	T	C	C	C	C	G	G	T	G	T	C	A	T
IA-3'recA3	PA55	T	C	C	C	C	G	G	T	G	T	A	A	T
IA-2/IB-3'recA1	PA7, 9, 13, 14, 18, 19, 23, 27, 32, 33, 34, 39, 40, 43, 44, 53, 54, 58, 64, 67, 68, 70, 72, 73, 76, 76b, 80, 82, 83, 83b, 83 t, 88, 89, 94, 95, 99, 104, 109, 113, 115	T	C	C	C	C	G	A	T	G	T	C	A	T
IA-2/IB-3'recA2	PA69	T	C	C	C	A	G	A	T	G	T	C	A	T
II-3'recA1	PA15, 22, 31b, 51, 56, 61, 62, 65, 85, 96, 98, 100, 108	C	C	C	C	C	G	G	C	A	T	T	G	T
II-3'recA2	PA84, 90, 91, 91b	C	T	C	C	C	G	G	C	A	T	T	G	T
II-3'recA3	PA57	T	C	C	C	C	G	G	C	A	T	T	G	T
II-3'recA4	PA36	T	C	C	C	C	G	G	C	A	T	C	G	T
III-3'recA	PA47, 48, 87	C	C	T	T	C	G	G	C	A	C	T	G	C

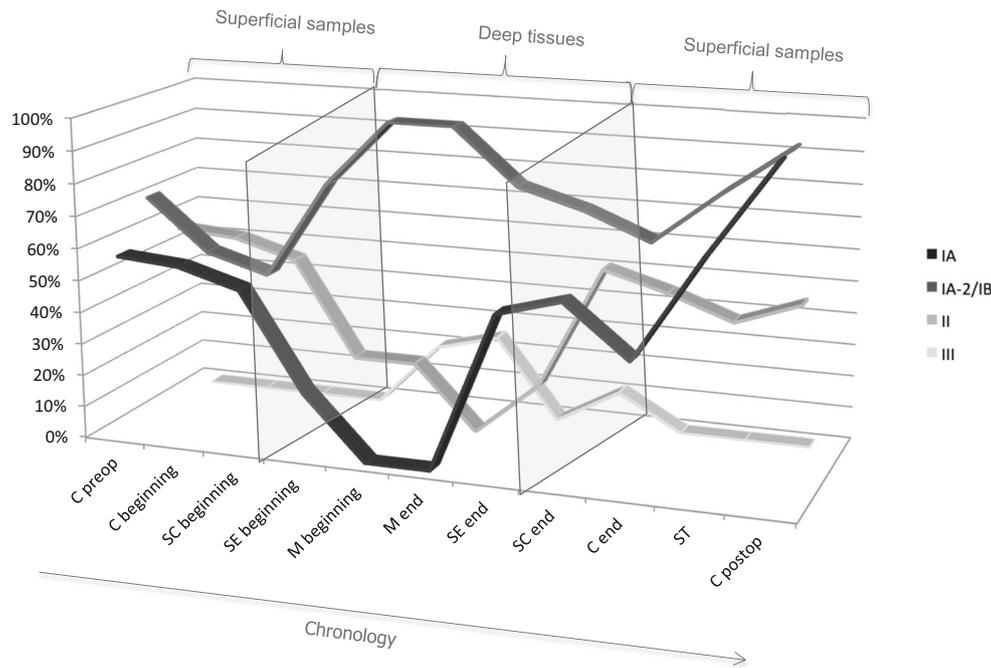


Fig. 5 Dynamics of the *P. acnes* phylotypes in samples chronologically collected during hospitalization and cardiac surgical procedures of 14 patients. The curves display the percentage of samples positive for each *P. acnes* phylotype according to the surgical stage and depth of sampling in the surgical site. *C preop* preoperative cutaneous sample, *C* cutaneous samples, *SC* subcutaneous samples, *SE* sternal edges samples, *M* mediastinal samples, *beginning* at the beginning of the surgical intervention,

end at the end of the surgical intervention, *ST* scar tissues, *C postop* postoperative cutaneous samples (3 months postdischarge). Superficial samples correspond to cutaneous, subcutaneous, and scar tissues. Deeper samples correspond to sternal edges and mediastinal samples both at the beginning and at the end of the surgical procedure. The arrow represents the chronological course of patient hospitalization

an interesting dynamics of the different phylotypes according to the depth of the wound and the step of surgical intervention (Fig. 5). Types IA and II displayed similar evolutions: they were present in superficial and low–deep tissues both at the beginning and at the end of the surgery, but they decreased in deep samples. Type IA greatly increased in postoperative samples such as scar tissues and in samples taken 3 months after surgery at a greater level than before surgery, while type II returned to its preoperative level. Type IA was also more present in sternal edges samples at the end than at the beginning of the intervention, suggesting peroperative colonization.

Type IA-2/IB presented an outstanding evolution, with a gradual increase with the depth of sampling, but also with the time of sampling in subcutaneous and cutaneous samples. This result suggested a particular resistance of phylotype IA-2/IB to the antimicrobial agents used in perioperative prophylaxis. However, both *P. acnes* IA and IA-2/IB cardiothoracic isolates were susceptible to antibiotics used in perioperative prophylaxis: cefamandole, vancomycin, and clindamycin.

Despite its low prevalence, type III displayed an increase in deep mediastinal tissues and subcutaneous tissues at the core phase of the surgery, but disappeared in superficial samples. Phylotypes IA and IA-2/IB were both predominant in scar tissues and on postoperative skin a long time after intervention, suggesting a specific predisposition to recolonize skin.

Discussion

P. acnes is an usual member of healthy skin microbiota, often considered a good bacterium devoid of pathogenicity [25]. However, its role in inflammatory processes in *acnes vulgaris* and in several infections has been established [11, 33–35]. In opposition to allopatric true pathogens, mutualistic bacteria are generally organized in species complex [36] and the comprehension of their opportunistic pathogenic behavior occurs through deciphering of the population structure inside the species complex. Most population genetic studies were focused on healthy skin and acne isolates, despite a wider the pathogenic spectrum of *P. acnes*. Deep or severe infections such as HAI were rarely studied, except for large collections of strains from failed hip replacements [26] and orthopedic implants [11]. Here, we aimed to determine if genetic subpopulations were specifically linked to specific sites or severe infections, as for the IA phylotype to acne. This hypothesis is supported by linkage disequilibrium analysis suggesting that the different phylogroups of *P. acnes* may occupy distinct ecological niches [25] and, therefore, may display different behaviors in different body sites. To meet our objective, we constituted a collection of natural isolates of *P. acnes* identified during severe infectious processes or from different origins in the routine exercise of hospital microbiology. The collection consisted mainly of strains from orthopedic and

cardiothoracic surgeries, as well as head and neck infections. Our collection is also original owing to strains from origins rarely represented or unrepresented in previous studies: peritoneal dialysis, gastric fluid in neonates, and otolaryngologic samples.

Several studies have already analyzed the *P. acnes* population structure by different methods, based first on serotyping and biotyping [37] and later by multilocus genetics and genomics [17, 19, 22, 23, 25, 26]. Comparative genomics based on 123,223 SNP nucleotides in the core regions highlighted that the *recA* classification of the strains was consistent with the major genomic clades [25]. The gene *tly* also has a recognized typing power [23, 25, 26]. In previous studies on large populations, the *recA* and *tly* genes showed good phylogenetic discriminative power and high genetic diversity index (0.67 each), making them good genetic markers [20, 25]. Moreover, despite the use of only two genes, we gained in allele variability because we analyzed larger sequences than that in major MLST schemes (available in <http://pacnes.mlst.net/> and <http://pubmlst.org/pacnes/>). Consequently, we found a greater genetic polymorphism than in other studies, with some polymorphic sites being present at the extremities of *recA* and *tly* sequences not included in previously published studies. The high polymorphism in the 3' part of the *recA* gene met our second objective, which was to develop a rapid and efficient tool (*recA* PCR-TTGE) to identify the major *P. acnes* phylotypes in complex communities without a first step of isolation by culture. The primary stratification of the *P. acnes* populations performed herein would be used as a basis for downstream MLST analysis on selected strains or selected phylotypes potentially associated with a particular type of deep infection.

However, deep MLST analyses [25] underscored the lack of specificity of *recA* due to the sharing of some type IB alleles with members of clonal complex 4 in phylotype IA-1 and with all type IA-2 members. Moreover, some *tly* alleles such as allele 8 were also shared between IA-2 and IB types [25]. Indeed, allele 8 is detected in 100 % of IB isolates and 97 % of IA-2 isolates [25]. In our phylogenetic tree, all the strains affiliated to the clade IA presented the *tly* allele 1. Therefore, considering the global congruence between the *tly* and *recA* trees, they certainly do not belong to IA-2 and probably corresponded to IA-1 isolates. For the alleles described herein, types IB and IA-2 could not be differentiated with certainty but type IA-2/IB differed clearly from type IA-1. The MLST schemes showed that IA-2 and IB underwent numerous recombination events involving most of the genes studied. Despite general linkage disequilibrium among phylotypes I, II, and III, the clonality of subtypes within phylotype I is less obvious. Particularly, IA-2 and IB phylotypes merged in split trees [25, 38] as the single clade named IA-2/IB with many parallelogram structures, indicating recombination events. The consequence is that lineages in phylotype I are not clearly

separated; particularly, IA-2 and IB should be considered as an undifferentiated group IA-2/IB whatever the typing scheme considered because of the high level of allele recombinations.

In the collection tested, we did not show a strict correlation between origins of infection and phylotypes. While previous studies showed that phylotypes II and III were rarely present in skin samples [19, 22, 24], we highlight their higher prevalence in deep infection sites. To our knowledge, we describe for the first time among strains from oropharyngeal sphere that about 35 % of them belong to type II. Not enough strains from blood cultures were included in this study but we observed a high prevalence of IA-2/IB and II. McDowell et al. [25] underlined the association of types IA-2/IB and II with medical devices, which are frequently the source of bacteremia in HAI. Both types IA and IA-2/IB are found in gastric fluids in neonatology. Therefore, we can hypothesize that the transmission from mother microbiota to newborn during delivery involves principally *P. acnes* type I.

As described previously [11], we found that phylotype I was the most often involved in orthopedic surgery, and type IA-2/IB (with 8 of the 12 isolates in the *recA* tree) more frequently than IA. Considering the lack of discrimination of *recA* underlined by McDowell et al. in 2012 [25], one can say that orthopedic surgery isolates more often belong to type IA-2/IB than to IA1. This was also the case for strains involved in cardiac SSI, unlike the data previously published by Davidsson et al. in 2012 [19], with a majority of type IA (only ten strains typed). These results suggest a particular tropism of type IA-2/IB for surgical wounds in orthopedic and cardiac surgeries, but not in neurosurgical infections, where phylotype IA was more often isolated.

Besides its genetic typing value, we studied the *tly* gene because it encodes a putative virulence factor that could be related to a particular pathogenic behavior. Except for three strains that probably encountered recombination events, *tly* and *recA* markers give congruent population structures, suggesting that *tly* coevolve with housekeeping genes. In spite of the general role of hemolysins in bacterial pathogenesis, comparative genomics prove that *tly* belongs to the core genome of *P. acnes* [25], but not to island-like genomic regions, encoding a variety of traits that differ between phylotypes [39]. McDowell et al. [25] hypothesized the importance of *tly* for both mutualistic and pathogenic lifestyles. Brzuszkiewicz et al. [39] concluded that the virulence of different *P. acnes* strains is not only determined by the phylotype-specific genome content but also by variable gene expression. For instance, despite the presence of *tly* in the *P. acnes* core genome, all strains do not express a hemolytic phenotype [26]. Moreover, *tly* hemolysin is not the only putative virulence factor able to induce hemolysis; several others like the group of CAMP factors and other cytotoxins, known to have this ability, have been identified in the *P. acnes* genome [4, 40].

Our results showed an interesting expression of hemolytic activity: the absence or very low hemolytic activity within phylotypes II and III, whereas about half of the IA strains and almost all IA-2/IB strains expressed hemolysis. Studies describing the hemolysis activities according to the genetic type in the *P. acnes* population are rare [41]. We found 70 % of phylotype I strains to be hemolytic, suggesting a potential role of hemolysin activity in infectious processes occurring in deep tissues. Concerning phylotype II, acne strains and deeper infection strains display similar hemolytic behavior, with about 10 % of hemolytic isolates in this study versus 12 % in the population of acne strains described by Kasprovicz et al. [41]. Besides hemolysis, biofilm formation is a virulence mechanism with potential importance in HAI [42]. *P. acnes* biofilm allows a latent growth [43, 44] that could lead to late-onset deep infections like SSI on foreign materials in orthopedic surgery or in endocarditis and prosthetic valves in cardiac surgery [8, 9, 11, 35, 43]. No correlation has been yet found between phylotypes and biofilm production, as all phylotypes contain isolates with different levels of biofilm production [43]. However, in sebaceous follicles, *P. acnes* biofilms are predominantly composed of types IA and II strains [45].

Finally, as for most opportunistic pathogens originating in the host microbiota, the virulence of *P. acnes* is difficult to decipher. Members of complex microbiota, such as human-associated microbiota, encounter complex relationships with host and other microbes. Therefore, the pathophysiology of the infection should take the microbiota dynamics into account as a major factor involved in infectious process. For this purpose, we propose a follow-up of the *P. acnes* diversity during the hospitalization courses of patients in cardiac surgery. Taking the opportunity for the high variability of the 3'-terminal region of the *recA* gene (13 variable positions in 333 bp), we developed an original culture-independent approach, the *recA* PCR-TTGE. This approach appears suitable to survey *P. acnes* populations during cardiac surgery procedures, allowing rapid screening of the main phylotypes involved in several samples. Our results showed that different phylotypes coexist in the same sample. This finding, which presented a certain novelty, is difficult to achieve when the *P. acnes* detection is based on culture because culture-based methods request the isolation of one pure bacterial colony. Phylotype IA-2/IB seems to be particularly adapted to colonize deep sternal and thoracic tissues (both sternal edges and mediastinum), as well as scar tissues after surgery. Type II was also very present at the different steps of hospitalization except in deep tissues, and did not increase during surgical intervention. These behaviors seemed to be not linked to particular resistance to antimicrobial agents used in cardiac surgical prophylaxis.

This study highlights a particular adaptation of phylotype IA-2/IB to cardiac surgery, in both the wound surgical

colonizing community and SSI. However, the strains isolated from cardiac SSI were obtained by cultural methods and did not reflect the whole *P. acnes* population initially involved in the sample. To confirm and complete the distribution scheme of *P. acnes* phylotypes in severe human infections, it would be necessary to increase the number of isolates from the different clinical sites and explore the dynamics of *P. acnes* phylotypes in diverse complex communities and their pathological variations. For this purpose, the specific culture-independent approach proposed herein would provide an efficient tool. Moreover, it also seems relevant to specify the phylotype(s) and/or the clonal complex (CC) present in surgical samples, thereafter to explore potential virulence characteristics involved in the pathophysiology of SSI.

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Conflict of interest The authors declare that they have no conflict of interest.

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