**Identification, typing and characterization of *Propionibacterium* strains from healthy
mucosa of the human stomach**

RUNNING TITLE: *Propionibacterium* strains from human stomach

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ABSTRACT

Forty two *Propionibacterium* isolates were recovered from biopsy samples of the gastric mucosa of eight out of 12 healthy people. Of these, 41 were identified as belonging to *Propionibacterium acnes*; the remaining isolate was identified as belonging to *Propionibacterium granulosum*. Repetitive extragenic palindromic (REP)-PCR typing suggested that up to four strains might be present in the mucosa of the same individual. Sequence analysis of either *recA*, *tly* or *camp5* genes of *P. acnes* isolates revealed two distinct phylogenetic lineages. As per the *recA*, most isolates belonged to type I, while the remainder of the isolates belonged to type II. Phenotypic analyses of representative isolates showed the different strains to have diverse biochemical properties. For example, large differences were seen in carbohydrate fermentation patterns, the results of qualitative and quantitative enzymatic profiling, and survival at acidic pH. In contrast, the patterns of resistance/susceptibility to a series of 16 antibiotics were rather similar, with no atypical resistances observed. The examined strains showed limited –if any– enzymatic activities that could be ultimately related to pathogenicity (lipolytic, proteolytic or haemolytic activity). This suggests that, in the gastric ecosystem, some *Propionibacterium* spp. genotypes and/or phenotypes can be considered true commensals.

KEY WORDS: Gastric microbiology, human stomach, propionibacteria, *Propionibacterium acnes*, *Propionibacterium granulosum*

1. Introduction

Propionibacterium species are non-spore-forming, Gram-positive, anaerobic-aerotolerant, pleomorphic rod-shaped bacteria, the fermentation end products of which include propionic acid. Besides the classical propionibacteria species from dairy products, *Propionibacterium acnes*, *Propionibacterium granulosum*, *Propionibacterium avidum*, *Propionibacterium propionicum*, and *Propionibacterium lymphophilum* are species typically found on the human skin and mucosa (Holland and Bojar, 2002), where they may exert either protective or harmful effects. However, in spite of this importance, scarce data are available for most species, except for *P. acnes*, the best known of the human-associated propionibacteria. Cutaneous *Propionibacterium* species might have genetic and biochemical similarity, occupying in consequence similar environments. Not surprisingly, thought at lower numbers, *P. granulosum* is usually found in close association with *P. acnes* (Dicksved et al. 2009; Saulnier et al. 2009).

P. acnes predominates over other microorganisms inhabiting the pilosebaceous follicles (Miura et al. 2010), and is a member of the normal microbiota of the oral cavity, the nostrils, conjunctiva, the external ear canal, and the upper respiratory and intestinal tracts (Bik et al. 2006; Saulnier et al. 2009). It is usually regarded as a strict anaerobe, although it grows better under low oxygen tension (Gribbon et al. 1994). In fact, *P. acnes* can tolerate 100% oxygen saturation, reflecting its ability to survive in a wide range of environments. *P. acnes* is well known for its role in acne vulgaris. Some authors suggest it contributes to the inflammatory phase of this condition (Dessinioti and Katsambas, 2010). Damage to host cells and tissues might occur via enzymes with degradative properties, including sialidases, neuraminidases, endoglycoceramidases and lipases (Brüggemann, 2005). The latter enzymes are thought to release free fatty acids, which in the skin irritate the follicular wall

and surrounding dermis. Immunogenic factors of *P. acnes* such as surface determinants, heat shock proteins and chemoattractants might then trigger inflammation (Vowels et al. 1995). *P. acnes* is also thought to be an opportunistic pathogen. It has been implicated in postoperative and device-related infections (Harris et al. 2005) as well as in a number of other conditions such as sarcoidosis, synovitis, pustulosis, endocarditis, endophthalmitis, hyperostosis and osteitis (Ishige et al. 2005; Leyden et al. 2001; Perry and Lambert, 2005). Infections generally occur in immunocompromised patients and newborns; they are less common in previously healthy individuals. However, evidence regarding the role of *P. acnes* as the causal agent of these diseases remains circumstantial.

P. acnes is also well known for its immunomodulatory properties. High levels of anti-*P. acnes* antibodies have been reported in individuals with acne (Ashbee et al. 1997). The induction of pro-inflammatory cytokines, interleukin IL-1a, IL-1b, IL-8 and TNF- α by *P. acnes* has also been reported (Vowels et al. 1995). The genome sequence of the species clearly shows it possesses many proteins involved in the ability to colonise and reside in human skin sites (Bruggemann et al. 2004), although no virulence determinants or pathogenicity factors have been identified. Thus, *P. acnes* is usually regarded as a disease-causing organism, although some authors indicate it to be a harmless commensal (Eady and Ingham, 1994).

The human stomach mucosa would appear to harbour a greater microbial diversity than originally anticipated (Bik et al. 2006; Monstein et al. 2000). Different species of lactobacilli and streptococci are among the majority populations seen (Bik et al. 2006; Ross et al. 2005). Although not among the dominant gastric microorganisms, *Propionibacterium* spp. has also been revealed by different culture-independent approaches (Dicksved et al.

2009; Monstein et al. 2000). Selected strains of all these bacterial groups could be of greater interest than others of intestinal origin for the design and formulation of probiotics. Their notable resistance to acidic conditions might be of use in maintaining the bacterial viability of probiotics during storage, in improving bacterial survival through the gastric passage, and be advantageous in the formation of biofilms (Holmberg et al. 2009), a key factor in gastric and intestinal colonisation (FAO/WHO, 2002).

This work reports the identification and genetic typing of *Propionibacterium* spp. strains isolated from the stomach of healthy adults. Phenotypic and biochemical tests were undertaken to characterize the gastric strains. In addition, activities related to pathogenicity and resistance to antibiotics were also sought to assess their potential to cause harm.

2. Material and Methods

2.1. Sampling, media and culture conditions

Gastric biopsies were aseptically collected from 12 healthy individuals undergoing routine upper gastrointestinal endoscopy by using an Olympus GIF-Q0165 gastroscope (Olympus Corporation, Tokyo, Japan) and a large (8 Fr) single-use, sterile biopsy forceps with a central bayonet (Boston Scientific Corporatin, Natick, USA). Before exploration, the gastroscope was washed and disinfected by immersion in a detergent solution with proteolytic enzymes (at 7%) and glutaraldehyde (at 2%). Biopsy samples were collected in sterile containers with a reduced saline solution (0.9% NaCl, 0.1% peptone, 0.1% Tween 80, and 0.02% cysteine) and transported to the laboratory in less than two hours after intervention. The biopsies were then washed several times in a phosphate buffered saline

(PBS) solution, drained, weighed, and thoroughly homogenized in Maximum Recovering Diluent (Scharlab, Barcelona, Spain). Dilutions of these samples were spread on agar plates containing the following media, MRS (Merck, Darmstadt, Germany) supplemented with 0.25% cysteine (Merck) (MRSC), Columbia Blood medium containing 5% defibrinated horse blood (CB; Merck), and Brain Heart Infusion medium (BHI; Oxoid, Basingstoke, Hampshire, UK). All plates were incubated anaerobically in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) at 37°C for up to 5 days. In order to rule out potential bacterial contamination the saline solution used to transport biopsy samples was spread on the same media. Non-inoculated plates, used as a second negative control, were also incubated in the same conditions.

2.2. Identification and genotyping of isolates

Visible colonies were selected, purified by subculturing on the same medium and identified at the species level by a combination of amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of the 16S rRNA amplicons.

Total genomic DNA from isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the DNA visualised by UV light after staining with ethidium bromide (0.5 µg ml⁻¹).

For ARDRA, the 16S rRNA genes were almost completely amplified by the polymerase chain reaction (PCR) technique using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and a 2 x Taq Master Mix (Ampliqon, Skovlunde, Denmark). Amplicons were purified

using GenEluteTM PCR Clean-Up columns (Sigma-Aldrich), digested with the restriction enzymes *Hae*III and *Hinf*I (Fermentas, Vilnius, Lithuania), and electrophoresed as above. When required, amplicons were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those in the GenBank database using the BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST/>), and to those held by the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>).

All isolates were genotyped by repetitive extragenic palindromic (REP)-PCR fingerprinting using primer BoxA2-R (5'-ACGTGGTTTGAAGAGATTTTCG-3'), as reported by Koeuth et al. (1995). Reproducibility studies of the REP-PCR technique showed a percentage of similarity of around 85%. Similarity of the patterns was expressed using Sorensen's correlation coefficient after clustering by the unweighted pair group method using arithmetic averages (UPGMA). Analysis of the *recA* gene with primers PAR-1 (5'-AGCTCGGTGGGGTTCGCTCATC-3') and PAR-2 (5'-GCTTCCTCATACCACTGGTCATC-3') was performed to assign the *P. acnes* strains to type I or type II, as reported by McDowell et al. (2005). Additionally, DNA sequence analysis of the two putative virulence genes *tly* and *camp5* was conducted using oligonucleotides and PCR conditions as previously described by McDowell et al. (2005) and Valanne et al. (2005), respectively. Sequences were compared to each other and to those deposited in the GenBank database. Similarity analysis was performed with the Neighbor-Joining algorithm using the program MEGA 4 (Tamura et al. 2007). For comparative purposes, representative published sequences from different genetic divisions associated with both health and infections (Lomholt and Kilian, 2010) were also included.

2.3. *Fermentation of carbohydrates*

Carbohydrate fermentation profiles of the different strains were established using the commercial API 50 CH system (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions.

2.4. *Enzymatic activities.*

Enzyme activities were assessed using the semiquantitative APIZYM system (bioMérieux). Sixty five millilitres of a cellular suspension corresponding to McFarland standard 5 were inoculated into each well of the API-ZYM strips, incubated for 4 h under anaerobic conditions at 37°C, and developed as recommended. Activities were expressed as nmol of substrate hydrolysed under the assay conditions.

2.5. *Acid resistance*

The ability of the strains to survive under acidic conditions was assessed through exposure of the cells to an acidified solution. Changes on the viability of cell suspensions (10^8 cells mL⁻¹) in sterile saline solution (0.5% NaCl) acidified with HCl to pHs ranging from 2.0 to 5.0 were determined by plate counting on MRSC agar after incubation at 37°C for 90 min.

2.6. *Antibiotic resistance/susceptibility profiles.*

Minimum inhibitory concentrations (MICs) were determined by microdilution in VetMIC™ plates for lactic acid bacteria (National Veterinary Institute of Sweden, Uppsala, Sweden) containing two-fold serial dilutions of 16 antibiotics. Colonies grown on MRSC agar plates were suspended in 2 mL of sterile saline (Oxoid) to obtain a density

corresponding to McFarland standard 2. This suspension was further diluted 1:15 with Mueller-Hinton medium (Oxoid) containing 0.5% glucose. One hundred microlitres of this were added to each well of the VetMIC™ plate, and the plates incubated at 37°C for 48-72 h. MICs were defined as the lowest antibiotic concentration at which no visual growth (pellet at the bottom of the well) was observed.

2.7. Presence of virulence factors

To determine the pathogenic potential of the strains, the presence of putative virulence-related traits was investigated using classical procedures. The production of haemolysins was analysed by plate assays on CB agar. The presence of haemolysis is indicated by the formation of clear zones surrounding the colonies. *Staphylococcus aureus* CECT 4520^T (Colección Española de Cultivos Tipo, Valencia, Spain) was used as a positive control. The possible interaction of pore-forming Christie-Atkins-Much-Peterson (CAMP) factors from the *P. acnes* strains with *S. aureus* sphingomyelinase C was tested as previously described (Valanne et al. 2005) using CB agar plates employing the *S. aureus* CECT 4520^T strain as an indicator. DNase activity was assayed on DNase agar plates (Merck). Proteolytic activity was determined on RCM plates containing 3% skimmed-milk powder (Scharlab). Lipolytic activity was scored on Reinforced Clostridial Medium (RCM; Merck) plates supplemented with 10 g L⁻¹ tributyrin (Sigma-Aldrich); all plates were incubated under anaerobiosis at 37°C for 3-4 days, the time at which the diameter of the clearing zone was measured. The production of urease was examined by growing the strains in urease test broth consisting of RCM supplemented with urea (20g L⁻¹) and phenol red (0.01g L⁻¹) (Sigma-Aldrich). Urease production was indicated by the change in the phenol red indicator.

3. Results

Viable bacteria of different species were recovered from the MRSC, CB and BHI agar plates for 10 of the 12 stomach mucosa samples examined. Counts ranged from 1×10^2 to 1×10^5 colony forming units (cfu) per gram of mucosa. Representative isolates from all plates were purified by subculturing and subjected to a polyphasic identification system; the latter included ARDRA profiling, the sequencing of the 16S rRNA gene, and the comparison of the sequences against those in databases. *Propionibacterium* spp. isolates were recovered in the different media from the mucosa of eight individuals. Surprisingly, of the 42 isolates identified as *Propionibacterium* spp., 41 belonged to the species *P. acnes*. The remaining isolate was identified as a *Propionibacterium granulosum*.

To assess the genetic diversity of the strains, all isolates were subjected to REP-PCR typing (Fig. 1). Statistical analysis of the results showed at least 12 genetic profiles with a similarity level below the percentage of reproducibility (85%) (Fig. 1); these can be considered to belong to different strains. Variability was observed even among isolates from the same individual, suggesting the presence of up to four strains in the stomach mucosa in some subjects. Representative isolates for these 12 profiles were subjected to a further genetic typing and to an exhaustive biochemical characterization to determine their genotypic, phenotypic and safety properties. Isolates with similar profiles but from different mucosa samples were also selected; thus, in total, 24 isolates were characterized, including the *P. granulosum* strain. The numbering of the strains, as represented in the tables and figures, includes species initials of the species, followed by the subject from which they were obtained and the code of the strain. Sequence analysis of *recA*, *tly* and *camp5* genes

from the 23 *P. acnes* strains showed two consistent and distinct cluster groups (Fig. 2). The number of polymorphic sites varied depending on the gene. Respect to *recA* gene, 19 strains harbour type I-specific sequences, while the remaining four (PA 1-3, PA 1-5, PA 6-4, and PA 8-2) contained all type II-specific polymorphisms. Further, a transition mutation (A for G) at position 897 of the *recA* gene corresponding to type IB (McDowell et al. 2008), was observed in nine of the 19 type I isolates. No type III-specific polymorphisms were recorded. Alignment of 777 bp of the *tly* genes revealed 22 differences in nucleotide positions and four allelic profiles, meanwhile in the *camp5* gene 19 polymorphic sites and four different alleles were encountered. Sequences were aligned with those from representative strains of the recent work of Lomholt and Kilian (2010). This allowed us to compare the relatedness at the DNA level of the gastric *P. acnes* isolates with those from clinical (infections) and non-clinical (environmental) sources. As can be seen in the derivative phylogenetic trees (Fig. 2), the gastric strains were clearly divided into two groups, one of which appears to include reference strains isolated from the oral cavity (such as CCUG45436 and CCUG 50655) and corresponded to *recA* type II isolates. The second group belonged to *recA* type I and included 19 strains. This group was further split into two subgroups, containing each a similar number of isolates; one of these subgroups seemed to be related to acne-associated strains, which belonged to *recA* type IA. The other subgroup, identified as *recA* type IB, included environmental sequences, no particularly associated with skin infections (Lomholt and Kilian, 2010)

The fermentation of 49 carbohydrates by the 24 selected strains was assayed using the API 50 CH system; Table 1 shows the results obtained. Rather wide phenotypic variation was observed among the different strains. Most fermented glycerol, D-arabinose, ribose, galactose, glucose, fructose, mannose, sorbitol, and N-acethyl-glucosamine, the utilization

of erythritol, adonitol, and mannitol was variable, and the fermentation of other carbohydrates was sporadic. Strengthening the genetic typing results, strains from the same individual showed different carbohydrate utilization profiles. Strains from different mucosa samples, however, sometimes showed identical or very similar profiles. It should be noted that the carbohydrate fermentation profiles do not always match those supposed specific for *P. acnes* *recA* type I or type II, the former –but not the latter– being able to ferment erythritol, ribose and sorbitol.

Although less than that observed for the fermentation of carbohydrates, variability among the different strains was also seen in terms of the enzymatic activities assayed using the API-ZYM method (Table 2). In general, the strains showed a limited number of weak enzymatic activities. The majority showed acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl- β -glucosaminidase activities. Weak alkaline phosphatase, esterase (C4), esterase lipase (C8), α -glucosidase and α -mannosidase activities were also scored for some strains.

The MIC values and antibiotic resistance-susceptibility patterns of 14 representative strains to 16 different antibiotics (all representatives of clinically important antimicrobial agents) were obtained by microdilution with the commercial VetMIC system. Table 3 shows the results obtained. Contrary to that reported for clinical *P. acnes* strains, low resistance levels were observed for most antibiotics, and analysis of the distribution of MICs among the different strains suggests they all are susceptible to most antibiotics. Significant variation was only observed with respect to kanamycin, streptomycin, and trimethoprim.

Table 4 shows the results regarding the presence of putative virulence and/or pathogenicity factors in the 24 selected strains. None of the strains showed urease activity in urea broth with phenol red as an indicator. Furthermore, DNase activity in DNase agar plates was never detected. Some slight haemolysis was seen for just two strains of the *recA* type IB in CB agar. However, haemolysis by either *P. acnes* or *S. aureus* was not affected when these were cultivated close to one another, excluding the involvement of CAMP-like factors in this activity. The production of proteases was scant; only three strains showed small (although clear) hydrolysis halos on RCM plates with skimmed milk. In contrast nearly all isolates showed halos of lipolysis in agar plates with tributyrin. Notwithstanding, a few strains showed faint halos, two (PA 1-3, PA 6-4) did not grow in the assay medium, and one (PA 1-5), which did grow, showed no halo. The latter three strains belonged to *recA* type II.

Species inhabiting the stomach ought to be highly resistant to acidic conditions. Analysis of this property was accomplished by incubating the strains in an acidic solution for 90 min and then plating. Counts were expressed as percentage survival compared to that recorded for control cells kept for the same time in a neutral solution. Strains maintained at pH 4.0 or higher showed no noticeable reduction in numbers, while they all were killed during treatment at pH 2.5 and 2.0. Fig. 3 shows large differences among the strains in the tolerance to pH 3.0. Reductions in counts of between 1 and 3 logarithmic units were commonly recorded at this pH.

4. Discussion

In this work, a series of 42 propionibacteria isolates from the stomach mucosa of healthy humans was identified by molecular methods, and then typed by REP-PCR. Of these, 41

isolates, corresponding to 23 different strains, were assigned to the species *P. acnes* and one to *P. granulosum*. These two species are usual members of the resident human microbiota in the sebaceous gland-rich areas of the skin (Miura et al. 2010), but also in conjunctiva, mouth, and intestines (Monstein et al. 2000; Saulnier et al. 2009). The *P. acnes* strains examined in this work mostly belonged to *P. acnes* *recA* type I, four belonged to type II and none to type III. Based on the fermentation of sugars or phenotypic profiling (Brüggemann, 2005; Higaki et al. 2000), the *recA* type II strains could not be distinguished from those of type I; this has been reported elsewhere (McDowell et al. 2005). Similar numbers of *recA* type IA and IB strains (as defined by McDowell et al. 2008) were found among the stomach isolates. These two groups corresponded with the clustering derived from *tly* and *camp5* gene analysis (Fig. 2). Sequence analysis of *tly* and *camp5* genes allowed us to allocate the gastric *P. acnes* strains in genetic divisions related or no with acne, following the recent study by Lomholt and Kilian (2010). According to these authors, the phylogenetic group termed as I-1a, that would include 10 of our strains, is strongly associated with acne, as compared to other divisions such as I-2 and II (which embraced 9 and 4 of the gastric strains, respectively). Strains from the latter groups seem to be associated with healthy skin and opportunistic infections. In our study, division II corresponded with the *recA* type II group. However, in spite of this associations, the role of the different genotypes in health and disease has is far from conclusive (McDowell et al. 2005; Lomholt and Kilian, 2010).

Wide phenotypic and genetic diversity was found among the strains from the different individuals. Diversity was even encountered between isolates from the same subject, and up to four different strains were identified in the same mucosa sample. REP-PCR analysis matched in general with the clusters derived from sequencing analysis of *recA*, *tly* and

camp5 genes, showing *recA* type II strains a closer genetic relationship among themselves than to type I strains (Fig. 1). This confirms previous findings suggesting the existence of consistent distinct phylogenetic groups within this species, as revealed by the use of genetic typing methods such as random amplification of polymorphic DNA (RAPD) or multilocus sequence typing (MLST) (Perry et al. 2003; McDowell et al. 2005; Lomholt and Kilian, 2010).

A gradual worldwide increase in the prevalence of antibiotic resistance in *P. acnes* has been observed (Nord and Oprica, 2006). This has been attributed to the major role of antibiotics in acne therapy (Eady et al. 2003). In Europe, combined resistance to clindamycin and erythromycin is highly prevalent in *P. acnes* isolates from patients with acne –from 50 up to 91% (Ross et al. 2003). In contrast, resistance to tetracycline was shown to be in the 0-26% range. Not surprisingly, a strong correlation between antibiotic resistance and the prescribing patterns of different countries exists (Ross et al. 2003). However, strains of this work seem all to be free of atypical resistances, those encoded by dedicated genes (Ammor et al. 2007). Resistance to aminoglycosides (kanamycin, streptomycin) in Gram-positives is thought to be due to intrinsic mechanisms. In fact, the lack of cytochrome-mediated transport is thought to be responsible for the strong resistance to aminoglycosides shown by most anaerobic and facultative bacteria (for a review see Ammor et al. 2007). Nevertheless, wide inter-strain variation in resistance levels to these antimicrobial agents, not linked to the presence or absence of dedicated antibiotic resistance genes, has been repeatedly reported in strains from different ecosystems (Ammor et al. 2007).

A vast array of degrading and host-interactive proteins located at the cell surface has recently been identified from the decoded genome of *P. acnes* (Brüggemann, 2005;

Brüggemann et al. 2004). Some of these proteins might be responsible for the phenotypic traits traditionally associated with pathogenesis (Dessinioti and Katsambas, 2010; Higaki et al. 2000). Production of CAMP factors and notable haemolytic and proteolytic activities that could ultimately be related with virulence were never detected by the classical methods used in this study. Moreover, clear differences in these properties were not scored among the different genetic types; only of note, was the scarce or absent lipolytic activity observed in three out of the four *recA* type II strains as compared with the rest of isolates. Furthermore, resistance to low pH (pH 3.0) was also shown to vary widely within the strains, independently of their genotype. Given that *Propionibacterium* species have been reported as a majority population in both healthy persons as well as in those suffering from many infections and inflammatory conditions (Eishi et al. 2010; Mihura et al. 2010), it would be of interest to determine the phenotypes and/or genotypes that render this bacterium either a commensal or pathogenic organism (Dessinioti and Katsambas, 2010; Mihura et al. 2010). The availability of the genome sequence of *P. acnes*, and soon that from other species, will allow post-genomic studies will help clarify whether the existing phenotypic properties are simply colonising traits or true virulence and pathogenic factors involved in the aetiology and pathogenesis of *P. acnes*-associated diseases.

5. Conclusion

Propionibacterium strains, mostly of the *P. acnes* species, were isolated as part of the dominant anaerobic cultivatable microbiota of mucosa samples from the human stomach. Wide phenotypic and genotypic diversity was encountered among the different strains, even among those isolates from the same individual. No clear pathogenic properties related to

the infectivity of this species were observed in the strains examined; however, genetic analyses proved some strains to be phylogenetically related with acne-associated strains.

Together, the present results suggest propionibacteria species might be habitual, innocuous inhabitants of the gastric environment. Though further research will be required to determine the precise role(s) of these bacteria in the gastric ecosystem it might be hypothesized about its contribution to the host health and well-being through their immunomodulatory activities. If this were the case, selected strains could be used as probiotics to counteract harmful bacteria such as *Helicobacter pylori*.

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FIGURE LEGENDS

Figure 1.- REP-PCR profiles of the 42 *Propionibacterium* spp. isolates obtained with primer BoxA2R. Below, dendrogram of similarity of the typing patterns clustered by the UPGMA method using the Sorensen's coefficient. Isolates having a coefficient higher than that of the reproducibility study (85%, indicated by a broken line) were considered to belong to different strains. Isolates having similar profiles but coming from gastric mucosa samples of different individuals were also selected for further characterization (indicated by a code of letters and numbers; see the text). Strains are all *P. acnes* except for PG 2-4, which is a *P. granulosum*. Strains of *P. acnes* type II are denoted by an asterisk.

Figure 2.- Neighbour-joining cluster analysis of individual partial DNA sequences of the *recA* (A), *tly* (B) and *camp5* (C) genes from 23 gastric *Propionibacterium acnes* strains. Published sequences of collection *P. acnes* strains (NCTC 10390 *recA* type II reference strain, NCTC 737 *recA* type I reference strain isolated from acne, CCUG 50480 from endocarditis, CCUG 32901 from blood, CCUG 27534 from urinary tract, CCUG 45436 from oral cavity and CCUG 50655 from mandibular gland) were included for comparative purposes. Strains of *P. acnes* type II are denoted by an asterisk.

Figure 3.- Survival of *Propionibacterium* spp. strains at pH 3.0 for 90 minutes. The results are expressed as the percentage of counts in MRSC as compared to a control dilution of the same strain maintained under the same conditions at pH 6.5. Strains are all *P. acnes* except for PG 2-4, which is a *P. granulosum*. Strains of *P. acnes* type II are denoted by an asterisk.

Figure

Figure 2

Figure 3

Table 1.- Carbohydrate utilization profiles of *Propionibacterium* strains from mucosa of the human stomach.

Strain ^a	Carbohydrate																									
	GLY	ERY	DAR	RIB	ADO	GAL	GLU	FRU	MNE	SBE	INO	MAN	SOR	MDM	MDG	NAG	SAL	MAL	LAC	MEL	SAC	RAF	GEN	TUR	TAG	GNT
PA 1-1	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 1-2	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 1-3*	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PA 1-4	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 1-5*	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 2-1	+	-	+	+	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 2-2	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 2-3	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
PG 2-4	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	+	-	-
PA 3-1	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 3-2	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-
PA 5-2	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-
PA 6-1	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 6-2	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 6-3	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	-	+	+	-	-	+	+
PA 6-4*	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 6-5	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	-	+	-	+	+
PA 7-1	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 7-2	+	-	+	+	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 7-3	+	-	+	+	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 8-1	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PA 8-2*	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
PA 8-3	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
PA 10-1	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-

Key of the compounds: GLY: glycerol; ERY: erythritol; DAR: D-arabinose; RIB: ribose; ADO: adonitol; GAL: galactose; GLU: glucose; FRU: fructose; MNE: mannose; SBE: sorbose; INO: inositol; MAN: mannitol; SOR: sorbitol; MDM: α-methyl-D-mannoside; MDG: α-methyl-D-glucoside; NAG: N-acethyl-glucosamine; SAL: salicin; MAL: maltose; LAC: lactose; MEL: melibiose; SAC: sucrose; RAF: raffinose; GEN: gentibiose; TUR: turanose; TAG: tagatose; GNT: gluconate.

None of the strains fermented L-arabinose, D-xylose, L-xylose, β-methyl-D-xyloside, rhamnose, dulcitol, amygdalin, arbutyn, esculin, cellobiose, trehalose, inulin, melezitose, stach, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, 5-keto-gluconate.

^aStrains are all *Propionibacterium acnes* except for PG 2-4, which is a *Propionibacterium granulosum*; asterisks mark *P. acnes* **recA** type II strains, all others were shown to belong to type I; grey shading indicates sugars used to distinguish between type I and type II strains.

Table 2.- Enzymatic activities as measured with the API ZYM system of *Propionibacterium* strains from mucosa of the human stomach.

Strain ^a	Enzymatic activity ^b							
	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Acid phosphatase	Naphtol-AS-BI-phosphohydrolase	N-acetyl- β -glucosaminidase	α -glucosidase	α -mannosidase
PA 1-1	0	0	0	15	10	10	0	0
PA 1-2	0	0	0	20	20	0	0	0
PA 1-3*	0	0	0	0	0	5	0	0
PA 1-4	0	0	0	10	10	10	0	0
PA 1-5*	5	0	0	35	30	2.5	0	0
PA 2-1	0	0	0	15	15	10	0	0
PA 2-2	2.5	0	0	30	30	10	15	10
PA 2-3	0	0	0	15	2.5	15	10	15
PG 2-4 ^b	35	10	2.5	40	35	0	2.5	0
PA 3-1	2.5	2.5	2.5	30	10	0	0	0
PA 3-2	5	2.5	0	20	0	0	0	0
PA 5-2	0	0	0	20	15	2.5	0	0
PA 6-1	0	0	0	20	15	2.5	0	0
PA 6-2	5	0	2.5	40	15	10	0	0
PA 6-3	0	0	0	15	5	5	0	0
PA 6-4*	10	0	0	40	15	10	20	0
PA 6-5	0	0	0	10	5	5	0	0
PA 7-1	5	0	2.5	40	30	10	0	0
PA 7-2	0	0	0	5	5	2.5	0	0
PA 7-3	2.5	0	0	35	30	10	0	0
PA 8-1	5	0	0	40	40	20	10	15
PA 8-2*	0	0	0	2.5	5	2.5	0	0
PA 8-3	15	0	0	40	40	15	15	15
PA 10-1	5	0	0	35	10	10	0	0

Leucine arylamidase, valine arylamidase, cysteine arylamidase, lipase (C14), trypsin, α -quimiotrypsin, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, and α -fucosidase activities were never detected.

^aStrains are all *P. acnes* except for PG 2-4, which is a *P. granulosum*; asterisks mark *P. acnes* **recA** type II strains, all others were shown to belong to type I.

^bUnits of activity are expressed as nmol of substrate hydrolyzed under the assay conditions.

Table 3.- Antibiotic resistance susceptibility profiles of representative strains of *Propionibacterium* strains from mucosa of the human stomach. MIC ($\mu\text{g mL}^{-1}$)

Strain ^a	Antibiotic															
	AM	PC	GM	KM	SM	NM	TC	EM	CL	CM	VA	VI	LZ	TM	CI	RI
PA 1-1	≤0.03	≤0.03	1	4	1	≤0.5	0.25	≤0.016	0.06	1	0.5	0.12	0.5	2	1	≤0.12
PA 1-3*	≤0.03	≤0.03	2	8	16	≤0.5	0.25	0.12	1	4	2	0.5	1	1	0.5	≤0.12
PA 2-1	0.06	≤0.03	2	8	4	1	0.5	0.03	0.06	0.5	0.5	0.12	0.5	1	0.5	≤0.12
PA 2-2	0.12	0.06	2	16	8	2	0.5	0.06	0.12	1	0.5	0.06	1	8	2	≤0.12
PG 2-4	≤0.03	0.12	1	8	4	≤0.5	0.5	≤0.016	≤0.03	0.5	0.5	0.06	0.5	32	0.5	≤0.12
PA 3-1	0.06	0.06	2	≤2	2	≤0.5	0.5	0.03	0.06	1	0.5	0.06	0.5	2	1	≤0.12
PA 3-2	0.12	2	≤0.5	≤2	2	≤0.5	0.25	0.12	0.25	4	1	0.25	1	1	≤0.25	≤0.12
PA 5-2	≤0.03	≤0.03	2	4	1	≤0.5	1	≤0.016	0.06	1	0.5	0.06	1	4	1	≤0.12
PA 6-1	≤0.03	≤0.03	2	4	1	1	0.5	0.03	0.06	1	0.5	0.06	0.5	2	1	≤0.12
PA 6-3	0.25	0.12	4	16	8	2	0.5	0.06	0.12	1	1	0.12	0.5	4	1	≤0.12
PA 7-1	≤0.03	≤0.03	2	4	≤0.5	2	0.5	≤0.016	≤0.03	0.25	0.5	0.06	0.12	0.25	0.5	≤0.12
PA 7-3	≤0.03	≤0.03	2	8	4	1	0.5	0.25	0.06	1	0.5	0.06	0.5	2	2	≤0.12
PA 8-2*	≤0.03	≤0.03	2	8	≤0.5	≤0.5	≤0.12	≤0.016	≤0.03	0.12	0.25	0.06	0.25	2	1	≤0.12
PA 10-1	≤0.03	≤0.03	2	8	2	1	0.25	≤0.016	0.06	0.5	0.5	0.06	0.25	1	1	≤0.12

Key of antibiotics: AM: ampicillin; PC: penicillin G; GM: gentamicin; KM: kanamycin; SM: streptomycin; NM: neomycin; TC: tetracycline; EM: erythromycin; CL: clindamycin; CM: chloramphenicol; VA: vancomycin; VI: virginiamycin; LZ: linezolid; TM: trimethoprim; CI: ciprofloxacin; RI: rifampicin.

^aStrains are all *P. acnes* except for PG 2-4, which is a *P. granulosum*; asterisk marks *P. acnes* *recA* type II strains, all others were shown to belong to type I.

Table 4.- Phenotypic, virulence-associated activities of *Propionibacterium* strains isolated from the mucosa of the human stomach.

Strain ^a	Activity ^b			
	Haemolytic	DNase	Lipolytic	Protolytic
PA 1-1	-	-	++	+
PA 1-2	-	-	++	(+)
PA 1-3*	-	-	Ng	-
PA 1-4	-	-	++	-
PA 1-5*	-	-	-	-
PA 2-1	-	-	+	-
PA 2-2	-	-	+	+
PA 2-3	-	-	+	(+)
PG 2-4	-	-	+	-
PA 3-1	-	-	+	-
PA 3-2	-	-	++	-
PA 5-2	-	-	++	-
PA 6-1	-	-	++	-
PA 6-2	-	-	++	-
PA 6-3	(+)	-	++	+
PA 6-4*	-	-	Ng	-
PA 6-5	-	-	++	-
PA 7-1	-	-	++	(+)
PA 7-2	-	-	++	-
PA 7-3	-	-	++	-
PA 8-1	(+)	-	++	-
PA 8-2*	-	-	++	-
PA 8-3	-	-	+	-
PA 10-1	-	-	++	(+)

^aStrains are all *P. acnes* except for PG 2-4, which is a *P. granulosum*; asterisks mark *P. acnes* **recA** type II strains, all others were shown to belong to type I.

^bNumber of crosses is related to the level of activity; in parenthesis, weak activities.
Ng, no growth observed.