Journal of Bacteriology and Parasitology

Arzumanian et al., J Bacteriol Parasitol 2016, 7:2
DOI: 10.4172/2155-9597.1000266

Research Article Open Access

Communities of Skin Propionic Bacteria: Cultivation and Antifungal Antagonistic Activity

Arzumanian V[1*], Zaborova V[2], Globa A[3] and Shmeleva O[1]

[1]*Laboratory of Fungal and Bacterial Physiology, Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia*

[2]*Department of Sports Medicine and Medical Rehabilitation of the Sechenov First Moscow State Medical University, Russia*

[3]*Institute of Agricultural Biotechnology, Moscow, Russia*

[*]**Corresponding author:** Vera Arzumanian, Laboratory of Fungal and Bacterial Physiology, Mechnikov Research Institute for Vaccines and Sera, Moscow, Russia, Tel: 8-916-495-20-21; Fax: 8-495-917-49-00; E-mail: veraar@mail.ru

Received date: March 02, 2016; **Accepted date:** March 18, 2016; **Published date:** March 21, 2016

Abstract

Many studies concerned the propionic bacteria of human skin report just about *Propionibacterium acnes*, whereas it is well known that the species diversity include at least three species - *P. acnes*, *P. granulosum* and *P. avidum*. Recently we have used real time PCR method, which allow detecting simultaneously the three species in skin washings of healthy people.

At the first step of the study we tried to isolate pure culture of *P. acnes*. From 7 swabs from healthy skin propionic bacteria one was selected for cultivation, because it contained according to real time PCR only cells of *P. acnes*. During the cultivation of this isolate in liquid medium probes were collected and again analyzed by real time PCR. All three species of propionic bacteria were detected in each phase of cultivation. In our opinion this fact may be a reason to check the collection strains named *P. acnes* towards their species "purity".

Antagonism of different propionibacteria against bacteria is well known, however so far nothing have published about their antifungal activity. The strain of *Candida albicans* was chosen as the test culture. Fungal cells were exposed with cell-free liquid of last stationary phase culture of propionic bacteria. About 59.4 % of alive fungal cells were killed during 2 hours when aliquot of *C. albicans* was combined with aliquot of bacterial cultural cell-free liquid. Propionic bacteria excreted at least two antifungal components–thermolabile and thermostabile. Small part of activity had molecular mass 3 ÷ 10 kDa, whereas the main part was contained in the fraction less than 3 kDa. Thus a new type of antagonistic activity of propionic bacteria was found out fungicidal activity, which confirm the existence of protective function of skin commensal microbiota.

Keywords: Skin propionic bacteria; Cultivation; Antifungal activity

Abbreviations:

Real time PCR: Real-time Polymerase Chain Reaction; DMEM medium: Dulbecco's Modified Eagle's Medium; YPD medium: Yeast Extract-Peptone-Dextrose Medium; CA: Complete Medium and Anaerobic Conditions; CM: Complete Medium and Microaerobic Conditions; CMAg: Complete Medium with Agarose and Microaerobic Conditions; DM: Defined Medium and Microaerobic Conditions; DMAg: Defined Medium with Agarose and Microaerobic conditions.

Introduction

According to present data, which are obtained by real time PCR method, the most abundant microorganisms–residents of human skin–are genera *Propionibacterium*, *Streptococcus* and *Staphylococcus* [1]. It is known that skin propionic bacteria are represented at least by species *P. acnes*, *P. granulosum* and *P. avidum* [2], although in old and modern medical articles usually referred only to *P. acnes*. During attempts to identify isolates obtained from host skin using traditional biochemical tests (indol formation, esculin hydrolysis and sucrose fermentation) we found out that most of isolates showed mixed results. Thereupon we have used the real time PCR method [3] in modification [4], which allowed to quantitatively identify all the three species of skin propionic bacteria. Use of this method allowed us to determine that propionic bacteria of more than 50 hosts are the mixtures of all three species simultaneously. Nevertheless the first task of the research was to isolate a pure culture of *P. acnes*.

It is known that members of resident microbiota are served for the defense of their ecosystem from foreign microorganisms. Earlier we have reported about mycocins–substances excreted by yeast *Malassezia* spp. and promoted the protection of the skin from opportunistic yeasts [5,6]. Proteomic analysis of proteins secreted by *P. acnes* showed the presence of hydrolases and adhesins, although the authors supposed a presence of antagonistic polypeptides (bacteriocins) [7]. Bacteriocins of propionic bacteria related to skin species - *Propionibacterium thoeniit* - were described earlier [8]. This bacterium is active towards some gram-positive and gram-negative bacteria and even some species of saprophytic mycelial and yeast fungi. Moreover it is known about bacteriocin-like substance–acnecin–from *Propionibacterium acnes*, isolated from oral cavity [9]. This polypeptide had molecular mass about 12 kDa and it lost its activity at 60°C and the action of protease, but demonstrated its bacteriostatic activity only towards *P. acnes* and closely related species-*Corynebacterium parvum*. It is common knowledge that classic propionic bacteria have three bacteriocins, at

that these substances do not have analogues in the data bases and their activity direct to related species/genera [10].

Aim of the research is to study of antagonistic activity and physiologic growth parameters of the human skin resident - *P. acnes*.

Materials and Methods

Strains and cultivation

Strains of propionic bacteria were obtained from their habitat- skin surface of people 22-65 years old with visually health skin. The wash-off was realized according to method of Korting [11] with some modification. Glass ring diameter 24 mm was hermetically pressed to the horizontally located skin surface at the middle of chest, and 1 ml of buffer consist from 0.1 M of potassium phosphate and 1% of Tween 80 (pH 7.9) was added into the ring. All skin into the ring was grinded with small sterile cotton plug during 1 min, whereupon liquid was collected. Aliquotes of the liquid were checked by qPCR and then seeded on Petri dishes with medium consist from: per 100 ml - 1 g casamino acids; 0.5 g yeast extract; 2.5 ml 40% sodium lactate; 1 ml Tween 80; 2 g agar; 20 μl 1% furazolidon. Dishes were incubated in atmosphere of 80-90% CO_2 during 4-7 days at 32°C up to appearance white semi-transparent colonies. Typical colonies were microscoped, seeded on the fresh medium and incubated the same manner. The procedure was repeated three or more times, after that cultures were used for cultivation in liquid medium.

Cultivation in liquid medium was carried out in 500 ml flasks contained 50 ml of medium. Before sterilization flasks were bubbled with CO_2 during 2 min, than sealed with silicon plugs. After sterilization furazolidon was added. For the microaerobic conditions the syringe needles with cotton swabs were introduced into plugs. Two types of media were used for cultivation-complete medium (see above) and defined medium DMEM supplemented with 1% Tween 80 ("PanEko", Russia). In two variants 0.1% of agarose was added. Flasks were seeded with culture from Petri dishes by syringe needle and incubated at 32°C without agitation. Aliquots of cultural fluid were periodically selected during growth period and their optical density was measured by spectrophotometer («Genesis 10S UV-Vis», USA) at 530 nm, width of cuvette 1 cm [4].

Extraction of DNA

Extraction of bacterial DNA was carried out with use of the DNA-extranEX-509 kit ("Synthol", Russia), namely, 100 μl of cell suspension was added to 5 μl 5 x 10^4 copies of Ptgt 2 plasmid in buffer pH 8 for the control of RNA run-out. Cell lysis was realized by 300 μl of 1% SDS solution with 20 mM Tris-HCl buffer (pH 8.0), 75 mM NaCl and 25 mM Na_2EDTA during 10 min at room temperature. Then proteins were precipitated by addition of 100 μl 7.5 M $NH_4(CH_3COO)$ and centrifugation at 12000 g during 2 min. DNA was sedimented from the obtained supernatant by the equal volume of isopropanol with the following centrifugation. The sediment of DNA washed by 75% ethanol and dried at room temperature. After that DNA was dissolved in 100 μl 10 mM Tris-HCl buffer (pH 8.0) at 65°C during 5 min, and the solution was used for PCR.

Real time PCR

For quantitative analysis of Ptgt 2 plasmid contained the insertion of unique sequence, the specific primers and probe labelled with fluorescent dye Cy5 were used [4]. The required oligonucleotides were synthesized by "Synthol", Russia. For the real time PCR the main mixture was prepared: 100 μl of buffer (500 mM KCl, 150 mM Trisc-HCl, pH 8.8, 0.5% glycerin, 0.1% Tween 20, 2.5 mM of dNTP mixture and 6.25 mM $MgCl_2$), 5 μl of Taq-polymerase with activity inhibiting antibodies (5 units/ μl), in 10 μl of primers (final concentration 0.20 μM) and 5 μl of sondes (final concentration 0.12 μM), and deionized water up to 200 μl of whole volume. Aliquots of the mixture (20 μl) were combined with DNA specimens (5 μl) and real time PCR were carried out in apparatus ANK-32 (Institute of analytical instrumentation technology, Russia) in following conditions: melting of DNA and activation of Taq-polimerase-95°C, 180 sec-1 cycle; (hybridization of primers and elongation of DNA chains - 60°C, 40 sec, melting-95°C, 15 sec)-45 cycles. For the construction of melting curves were used the solutions of oligonucleotides-amplicons synthesized for each species of bacterium with known concentrations 10^3-10^6 copies/μl.

The obtained results were normalized upon the RNA yield percent, which was determined according to yield of Ptgt-2 plasmid preliminary added to the specimens. Quantity of bacterial cells per specimen was calculated taking into account of known quantity of operon 16sRNA copies-3 copies per cell [2] and evaluated as quantity of cells in 100 μl of specimen.

Estimation of antagonistic activity

For the analysis the stationary phase liquid culture of propionic bacteria grown in compete medium with agarose at microaerobic conditions was used. Cells were separated by centrifugation during 5 min at 10000 g, supernatant was filtrated through cellulose membrane 0.22 μm and freezed in small portions (specimen A). The strain of *C. albicans* № 4387 from laboratory collection was grown on Petri dishes with YPD medium up to the end of exponential phase and used as indicator culture. Aliquot (30 μl) of *C. albicans* cells suspension (10^8 cells per ml) was combined with aliquot (300 μl) of specimen A and incubated at 32°C during 2 h (in control variant specimen A was combined with physiological solution). After the incubation suspension was centrifuged and cells pellet was stained by 0.2 mM bromocresol purple solution with 0.1 M sodium phosphate buffer pH 4.6 during 45 min at 32°C, centrifuged again and the pellet estimated microscopically [12]. Percent of cells killed during incubation with specimen A was calculated taking into account the dead cells in the control variant. Portions of specimen A were filtrated through membranes «Microcon» (Germany) with pore size of 3, 10 and 30 kDa, than the filtrates antagonistic activities were analyzed the same manner. Besides this one portion of specimen A was autoclaved at 120°C and pressure 0.5 atmospheres and its activity was analyzed too.

Results

The speciation of propionic bacteria of 7 isolates obtained from skin of 7 healthy hosts demonstrated in table 1. From 7 cultures one - № 4 - was chosed, because according to real time PCR data, it consisted from one species - *P. acnes*. This strain was passed on Petri dishes, than seeded into flasks with liquid media for the cultivation at different conditions (Figure 1, Table 2). From the growth curves and calculated physiological parameters it is obvious that in anaerobic conditions even in complete medium (CA) growth was minimal, whereas Microaerobic atmosphere (CM) lead to improvement of all growth characteristics-decrease of lag-phase and doubling time, increase of maximal growth rate and protein yield. Furthermore addition of 0.1%

agarose as the substrate for adhesion (CMAg) considerably improved bacterial growth too. Defined medium without agarose (DM) did not support any growth, but supplemented with agarose (DMAg) provided significant shortening of lag-phase and increase of growth rate and protein even compare with complete medium CMAg.

No of specimen	Containing of propionic bacteria in human skin specimens, cells per 100 µl		
	P. acnes	*P. granulosum*	*P. avidum*
1	1320	0	594
2	212270	33470	0
3	495	957	5874
4	57970	0	0
5	124773	18010	5544
6	7128	0	4983
7	117615	2030	0

Table 1: Detection of different species of propionic bacteria in human skin swabs by real time PCR method.

Name of variant	Type of medium for the cultivation	Aeration regime	Lag-phase, days	Maximal growth rate, 1/ day	Doubling time, days	Biomass in stationary phase, mg of protein/ml
CA	Complete	Anaerobic	13	0.029	23.32	0.055
CM	Complete	Microaerobic	10	0.058	11.96	0.104
CMAg	Complete + agarose	Microaerobic	10	0.124	5.60	0.186
DM	Defined	Microaerobic	-	0	0	0
DMAg	Defined + agarose	Microaerobic	2	0.141	4.93	0.387

Table 2: Physiological parameters of propionic bacteria growth at different regimes of cultivation.

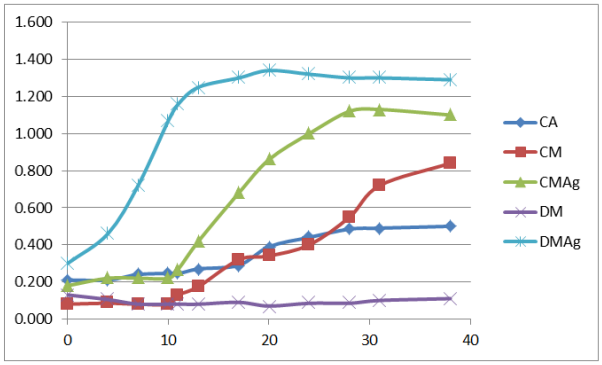

Figure 1: Growth of skin propionic bacteria in liquid media: Abscissa - days of cultivation; ordinate- optical density at 530 nm.

From the culture CMAg at 1st, 9th, 15th, 21st, 29th and 35th days of cultivation probes for real time PCR were collected (Figure 2). As it turned out, in all probes the three species of propionic bacteria were detected, but in different proportions depends on growth phase. At the exponential phase the prevalent species was *P. acnes*, whereas later *P. granulosum* was dominated. Therefore despite the multiple attempts to obtain monoculture of propionic bacterium *P. acnes*, the initially "pure" culture appeared to be the mixture of three species. Obviously in the process of initial isolate passing in the followed cultures was recovered the typical presence of three species.

As the strain- producent of antagonistic activity- an isolate of propionic bacteria were chosed. The isolate was obtained from the skin of healthy man and consisted from species *P. acnes* and *P. avidum* at ratio 5:1. The isolate was cultivated up to the late stationary phase in the complete medium CMAg. After that the antagonistic activity of secreted substances towards *Candida* yeast was measured, because just this fungi is often reason of skin mycoses. From the results shown in table 3 one can conclude, that the antagonistic activity of skin propionic bacteria towards yeast *C. albicans* really exist. Moreover, as far as the percent of dead cells was estimated by the dying with bromcresol purple, which insert through the membrane pores of killed cells, one can assume about the mechanism of action of the bacteriocins–they destroyed a membrane integrity.

At the mentioned concentration of yeast suspension-10^7 cells/ml–59.4% of cells during 2 h were killed by bacterial specimen A. Based on the molecular filtration data it is clear that antifungal activity of

propionic bacteria localized in two fractions–small part in diapason 3 ÷ 10 kDa, and the general part in the low molecular fraction, i.e., ≤ 3 kDa. The activity evidently consists from two components–thermolabile (maybe polypeptide) and thermostable. Earlier described acnecin [9] had very close spectra of action - only two related species and lost the bacteriostatic activity at high temperature and action of proteases.

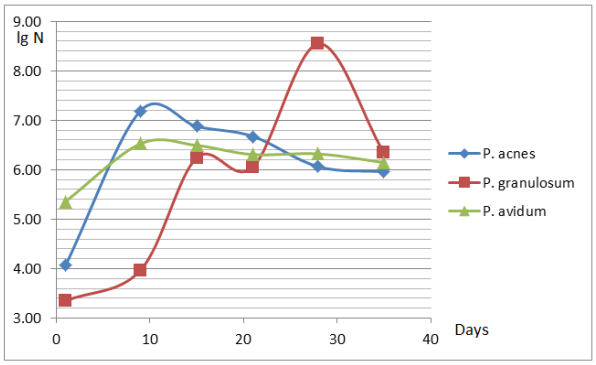

Figure 2: Ratio between three species of skin propionic bacteria measured by real time PCR method in different phases of culture grown in liquid complete medium (CMAg).

Type of specimen	Fungicidal activity, % of killed cells of *C. albicans* test-culture
Specimen A (cell-free cultural liquid after cultivation of propionic bacteria in liquid complete medium CMAg)	59.4 ± 2.7
Autoclaved specimen A	34.5 ± 3.5
Specimen A filtered through membrane 3kDa	54.9 ± 1.8
Specimen A filtered through membrane 10 kDa	61.7 ± 2.9
Specimen A filtered through membrane 30 kDa	60.6 ± 3.1

Table 3: Antagonistic activity of substances secreted by propionic bacteria towards yeast *C. albicans*.

Conclusion

As directed by the first step of our study we should isolate pure culture of *P. acnes*. Unfortunately we could not do it, because initially "pure" isolate after passing on solid and in liquid media recovered to mixture consisted (according to real time PCR data) from three species of propionic bacteria-*P. acnes*, *P. granulosum* and *P. avidum*. Thereupon a question is: are the strains from human skin, which were described in literature as *P. acnes* before the method of real time PCR was appeared, were really monocultures of the species or we can consider them only as mixture with the prevalence of one species? In our case we can surely conclude that we deal with communities of propionic bacteria. This fact may be a reason to check the collection strains named P. acnes towards their species "purity".

Antagonism is widely spread among microorganisms, but we have little information about this phenomenon in skin propionic bacteria. Some data concerned the activity of the bacteria against related bacterial species, although since the present time nothing was known about their activity towards opportunistic fungi. Here we present one more type of antagonistic activity of skin propionic bacteria-fungicidal activity, which destroyed a membrane integrity, consist from thermolabile and thermostable fractions and have low molecular mass. The data obtained confirm the idea about protective function of commensal skin microbiota against opportunistic species [13].

Acknowledgement

Authors express their thanks to Tamara Artemieva and Liubov Butovchenko for technical support and permanent care.

References

1. Gao Z, Perez-Perez GI, Chen Y, Blaser MJ (2010) Quantitation of Major Human Cutaneous Bacterial and Fungal Populations. J Clin Microbiol 48: 3575-3581.
2. Noble WC (1981) Microbiology of human skin. Lloyd-Luke, London.
3. Miura Y, Ishige I, Soejima N, Suzuki Y, Uchida K, et al. (2010) Quantitative PCR of Propionibacterium acnes DNA in samples aspirated from sebaceous follicles on the normal skin of subjects with or without acne. J Med Dent Sci 57: 65-74.
4. Globa AG, Alekseev YI, Arzumanyan VG, Zaborova VA, Guridov AA (2013) The use of real time PCR for quantitative determination of some propionic bacteria inhabiting the human skin. Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry 7: 175-177.
5. Arzumanian V (2003) Yeast Malassezia: Taxonomy, identification, significance in human ecology and pathology: New in systematics and nomenclature of fungi. (Eds. Diakov Y, Sergeev Y), "Meditsina dlia vseh", Moscow.
6. Arzumanian VG, Sergeev AY, Shelemekh OV, Ojovan IM, Serdiuk OA (2009) Antagonistic activity of Malassezia spp. towards other clinically significant yeast genera. Bulletin of Experimental Biology and Medicine 148: 410-415.
7. Holland C, Mak TN, Zimny-Arndt U, Schmid M, Meyer TF, et al. (2010) Proteomic identification of secreted proteins of Propionibacterium acnes. BMC Microbiology 10: 230.
8. Lyon WJ, Glatz BA (1991) Partial Purification and Characterization of a bacteriocin produced by Propionibacterium thoeniit. Appl Envir Microbiology 57: 701-706.
9. Fujimura S, Nakamura T (1978) Purification and properties of a bacteriocin-like substance (acnecin) of oral Propionibacterium acnes. Antimicrob Agents Chemother 14: 893-898.
10. Faye T, Holo H, Langsrud T, Nes IF, Brede DA (2011) The unconventional antimicrobial peptides of the classical propionibacteria. Appl Microbiol Biotechnol 89: 549-544.
11. Korting HC, Loferer S, Hamm N (1991) The detergent scrub method for quantitative determination of Malassezia furfur on chest and back skin: comparative evaluation of three different media. Mycoses 34: 267-271.
12. Kurzweilova H, Sigler K (1993) Fluorescent staining with bromocresol purple: a rapid method for determining yeast cell dead count developed as an assay of killer toxin activity. Yeast 9: 1207-1211.
13. Christensen GJ, Brüggemann H (2014) Bacterial skin commensals and their role as host guardians. Benef Microbes 5: 201-215.