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Hydrophobicity and Biofilm Formation of Lipophilic Skin Corynebacteria

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A b s t r a c t

Lipophilic corynebacteria isolated as natural flora of human skin were examined. Among 119 assayed strains 94% presented a hydrophobic cell surface and 75.6 % were able to form biofilms. These attributes, as well as aggregation in liquid media, were statistically connected with each other and promote the developing of biofilms on solid surfaces. This was characteristic of all the lipophilic *Corynebacterium* species found on human skin that were examined in this study. *C. jeikeium* and CDC group G2 strains dominated in this population, and they could be responsible for investigated features in the whole lipophilic skin bacterial population. These two groups are the most common coryneform bacteria isolated from nosocomial infections and these attributes most likely promote them to cause opportunistic infections.

K e y w o r d s: biofilm, *Corynebacterium*, hydrophobicity

Introduction

Lipophilic species of *Corynebacterium* present a stable position among bacteria forming resident flora of the skin. Although their occurrence is not numerous, their specific contribution in this habitat is quantitatively stable. In this group of skin residents the most often represented taxa are *C. jeikeium*, CDC groups G1 and G2 and *C. afermentans* subsp. *lipophilum*. Lipophilic corynebacteria stay in dynamic equilibrium with coagulase-negative staphylococci (Roth and James, 1989; Tancrède, 1992; Kaźmierczak *et al*., 2005).

Many reports show that these bacteria, apart from being a natural flora, can participate in severe opportunistic infections. Such infections cause treatment difficulties because many strains are multiresistant to antibiotics (Coyle and Lipsky, 1990; Williams *et al.*, 1993; Bayston *et al*., 1994; Funke *et al*., 1997). Ability to develop acquired hospital infections is strongly connected with long term survival in a hospital environment, easy selection of resistant strains or medical device colonization due to biofilm formation. Our knowledge concerning coryneforms in these fields is incremental. According to the estimation by the Centers for Disease Control and Prevention about 65% of bacterial infections in humans are connected with the formation of bacterial biofilms (Cvitkovitch *et al*., 2003). Many physico-chemical features of surfaces of colonised devices and bacterial cells affect biofilm creation. The main ones are: specific cell wall surface adhesion, bacterial hydrophobicity and the ability to synthesise extracellular slime substances (ESS) (Sutherland, 2001).

The aim of this study was to analyse bacterial cells hydrophobicity and the ability of the lipophilic *Corynebacterium* strains to form biofilms on artificial surfaces.

Experimental

Material and Methods

The population of 119 strains of *Corynebacterium* isolated from human skin on the back and the forehead was investigated: *C. afermentans* subsp. *lipophilum* (10 strains), *C. diphtheriae* var. intermedius (3 strains), *C. jeikeium* (29 strains), *C. kroppenstedtii* (3 strains), *C. urealyticum* (4 strains), CDC group F1 (5 strains), CDC group G1 (15 strains), CDC group G2 (34 strains), *Corynebacterium* spp. (16 strains). The number of strains used in experiments reflected particular species participation within *Corynebacterium* genus population on skin (Kaźmierczak *et al*., 2005). All strains were cultured and stored on TYT80 medium (Kaźmierczak and Szewczyk, 2004).

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Microbial adherence to hydrocarbons was determined according to Rosenberg *et al.* (1980) method (MATH test). Strains were grown on nutrient broth (POCH) supplemented with 0.1% Tween 80 at 37°C for 72 h (orbital shaker – 90 cycles/min). Cells were harvested, washed twice with demineralised water and resuspended in a 10 mM potassium phosphate buffer adjusted to pH 5 with HCl.

Optical densities of bacterial suspensions were standardized at 600 nm to approximately 3×10^8 CFU/mL (A_0). 150 µL of *p*-xylene was added to a glass tube with 3 mL of bacterial suspension which was then vortexed vigorously for 60 s. After 15 minutes, phases were separated and aqueous phase was carefully removed to measure the optical density of cells remaining in suspension (A_t). Hydrophobicity was calculated as the percentage of cells partitioning in the hydrocarbon phase. The % of *p*-xylene partitioning was determined by the formula: = $(A_0 - A_t/A_0) \times 100$. Strains were tested in triplicate. When the mean adherence to *p*-xylene was ≤ 30%, strains were considered hydrophilic, those whose values were > 30% were considered hydrophobic, and among them, highly hydrophobic strains gave values ≥ 70%.

Quantification of biofilm development was performed according to modified Tsai method (Tsai *et al.,* 1988). Strains were grown in TSB broth (Biocorp) supplemented with 0.03% Tween 80 (ICN Biomedicals) at 37°C for 3 hours on an orbital shaker and incubated stationary for 93 hours. Culture medium was decanted and the biofilm formed on glass surface was washed three times with demineralised water, dried at room temperature and fixed using Carnoy's solution (absolute ethanol, chloroform, glacial acetic acid 6 : 3 : 1) for 15 s. Fixed biofilm was stained with 0.1% safranine for 1 h, washed three times with demineralised water, dried and hydrolysed with 0.2 M NaOH at 85°C for 1 h. The optical density was measured at 540 nm with a Microplate Reader 680 (Bio Rad). All strains were assayed in triplicate. Biofilm-producing strains were arbitrarily defined to have mean $A_{540} > 0.06$. The biofilm-producing strains were classified as average ($0.06 < A_{540} < 0.2$) and abundant biofilm-producers ($A_{540} > 0.2$).

Results

Many investigated strains presented aptitude for spontaneous auto-aggregation when growing in nutrient broth. 23.5% of strains formed compact clusters during growth, sometimes suspended in completely clear medium. We could clasify these strains as rough. This observation led to an assumption about their strong hydrophobicity. In Table I the percentage of rough and smooth strains in particular species are shown.

Table I
Percentage of rough strains in particular species

Species	Number of strains	Rough strains (%)	Smooth strains (%)
C. afermentans subsp. *lipophilum*	10	50.0	50.0
C. diphtheriae var. intermedius	3	0.0	100.0
C. jeikeium	29	27.6	72.4
C. kroppenstedtii	3	0.0	100.0
C. urealyticum	4	25.0	75.0
CDC group F1	5	40.0	60.0
CDC group G1	15	13.3	86.7
CDC group G2	34	23.5	76.5
Corynebacterium spp.	16	12.5	87.5

Table II
Hydrophobic strains within species and in the whole lipophilic corynebacteria population resident on the skin

Species	Number of strains	Number of hydrophilic strains	Number of hydrophobic strains*	Hydrophilic (% of all 119 strains of tested population)	Hydrophobic (% of all 119 strains of tested population)
C. afermentans subsp. *lipophilum*	10	0	10 (3)	0	8.4
C. diphtheriae var. intermedius	3	0	3	0	2.5
C. jeikeium	29	4	25 (3)	3.4	21.0
C. kroppenstedtii	3	0	3	0	2.5
C. urealyticum	4	0	4	0	3.4
CDC group F1	5	0	5	0	4.2
CDC group G1	15	1	14	0.8	11.8
CDC group G2	34	0	34 (7)	0	28.5
Corynebacterium spp.	16	2	14 (5)	1.7	11.8
All strains investigated	119	7	112 (18)	5.9	94.1

* in brackets – number of strains presenting strong adhesion

Table III
Strains able to form biofilms within species and in the lipophilic corynebacteria population resident on skin

Species	Number of strains	Number of biofilm-negative strains	Number of biofilm producing strains*	Biofilm-negative (% of all 119 strains of tested population)	Biofilm-positive (% of all 119 strains of tested population)
C. afermentans subsp. *lipophilum*	10	2	8 (1)	1.7	6.7
C. diphtheriae var. intermedius	3	2	1	1.7	0.8
C. jeikeium	29	8	21 (1)	6.7	17.6
C. kroppenstedtii	3	2	1	1.7	0.8
C. urealyticum	4	1	3 (1)	0.8	2.5
CDC group F1	5	0	5 (1)	0.0	4.2
CDC group G1	15	3	12	2.5	10.1
CDC group G2	34	7	27 (4)	5.9	22.8
Corynebacterium spp.	16	4	12 (2)	3.4	10.1
All investigated strains	119	29	90 (10)	24.4	75.6

* in brackets – number of strains forming abundant biofilms

The MATH method based on the degree of adherence to the interface of hydrocarbon (*p*-xylene) and water (buffer pH 5) showed that more than 94% of strains were hydrophobic. Eighteen strains presented very strong adhesion to hydrocarbons and only seven were hydrophilic.

The number of hydrophobic strains present in particular species is shown in Table II. Strongly hydrophobic strains belonged to *C. afermentans* subsp. *lipophilum, C. jeikeium,* CDC group G2 and many strains of *Corynebacterium* which were not identified to species level. The data presented help to estimate the influence of particular species on the general hydrophobicity of all strains forming the population of corynebacteria inhabiting human skin.

In the biofilm formation experiments 90 out of the 119 strains (75.6%) developed biofilm (Table III). Ten of them produced biofilm abundantly. Strains able to form biofilm came from all species. The biofilm-forming ability of the whole population of corynebacteria living on the skin depends on the two most numerously represented taxa CDC group G2 (22.8% producers) and *C. jeikeium* (17.6 %). The correlation between cell surface hydrophobicity and their ability to form biofilm was analysed. Among all MATH tested hydrophobic strains (112), one hundred were also able to form biofilms. Almost all strongly hydrophobic strains formed biofilms (94.4%). Statistical analysis by chi^2 test revealed strong interdependence of these features in the investigated strains ($p<0.05$).

Biofilm formation was also a trait of strains of corynebacteria that aggregated during growth in liquid media (Figure 1). The correlation of these two features was also statistically significant (chi^2 test; $p<0.05$).

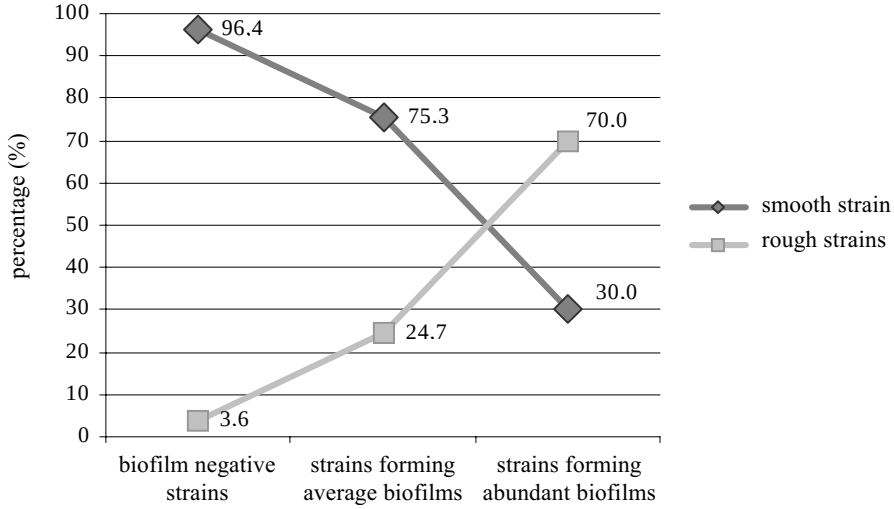

Fig. 1. Biofilm formation and rough type growth in liquid media

Discussion

The emergence of opportunistic nosocomial infections is commonly known. These infections are frequently caused by opportunistic pathogens originating from natural physiological endogenous flora. So, characterization of well-known and newly described opportunistic pathogens is an incessant challenge for researchers. Among coryneform bacteria living on human skin almost 85% are lipophilic. This population is a very constant component of the natural flora of the skin in all examined samples. *C. jeikeium* and CDC group G2 predominate (Kaźmierczak and Szewczyk, 2004; Kaźmierczak *et al*., 2005).

The pathogenicity of some lipophilic species has already been characterized (Funke *et al*., 1997). The attention of authors has concentrated on the most often isolated *C. jeikeium* causing septicaemia and various infections in surgical patients undergoing treatment using different medical devices. According to Williams *et al.* (1993) CDC group G are the second most frequently occurring corynebacteria isolated from opportunistic infections. *C. urealyticum* was connected with urinary tract infections and *C. afermentans* subsp. *lipophilum* was isolated from blood specimens and abscesses. Most clinically isolated strains were multiresistant to antibiotics (Funke *et al*., 1997).

The constant occurrence and domination of these species of corynebacteria on human skin and their frequent isolation from nosocomial infections must be closely related and must be also an effect of their special relationships with skin and artificial surfaces *i.e.* hydrophobicity and other features that promote biofilm formation. Hydrophobicity plays a major role in bacterial adhesion (Absolom, 1988; Bendinger, 1993) and facilitates spread of the organism by bacteria ingested by phagocytes. Hydrophobicity increases and biofilm formation becomes more intensive under conditions of developing infection in the presence of serum (Olson *et al*., 2002) and iron limitation (Baldssarri *et al*., 2001; Moreira *et al*., 2003). Knowledge about these features in corynebacteria mainly concerns the adhesion ability of pathogenic *Corynebacterium diphtheriae.* This is attributed to their hydrophobicity connected with the structure of the cell wall, specific for the whole genus. This contains different types of long-chain corynomycolic acids; chain length ranges from 22 to 36 carbon atoms (Bendinger *et al*., 1993). There is no data concerning skin habitants, especially lipophilic species. Lipids responsible for a barrier function of the skin make an excellent base for adherence of hydrophobic bacteria. The lipid layer is also the key to colonization of the skin as these bacteria need lipids for growth. Almost all lipophilic strains examined in this research were hydrophobic. The ability to form biofilm was presented by almost 76% of strains. The most hydrophobic and active in biofilm formation were *C. jeikeium* and CDC group G2. As they are the most numerous taxa represented among corynebacteria naturally inhabiting skin, the probability of contamination of wounds, implants and different medical devices must be very high.

Hydrophobicity and biofilm formation seems to be crucial for both skin colonization and developing opportunistic hospital infections. *Staphylococcus epidermidis* is well known as the main etiologic factor of these kind of infections. This species is recognized, among other staphylococci important for human pathology, as the best biofilm producer and often develops biofilms on the surfaces of intravenous catheters and other medical devices. Mixed populations of corynebacteria and staphylococci may form a mutually supporting "organism" which can efficiently oppose immune response and antibiotic action (Bayston *et al*., 1994). The presence and role of *Corynebacterium* strains in this kind of infection is still seldom noticed, due, as Funke *et al.* (1997) stated, to the relatively small database of knowledge gathered concerning these difficult to cultivation microorganisms. Their role must be significant considering our data presented here.

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