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Physicochemical Cell Surface and Adhesive Properties of Coryneform Bacteria Related to the Presence and Chain Length of Mycolic Acids

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The presence and chain length of mycolic acids of bacteria of the genera *Corynebacterium*, *Rhodococcus*, *Gordona*, *Mycobacterium*, and *Arthrobacter* and of coryneform bacteria containing a type B peptidoglycan were related to the cell surface hydrophobicity of the bacteria, which in turn was related to adhesion of the cells to defined surfaces such as Teflon and glass. The origin of the overall negative charge of these bacteria is discussed.

Bacterial adhesion is controlled by the hydrophobicity (i.e., cell-water contact angle Θ [measured in degrees]) as well as the negative electrokinetic potential of the cell surface and substratum (26). The effect of repulsive electrostatic interactions on adhesion decreases with increasing hydrophobicity (26). Other studies also suggest that hydrophobicity plays a major role in adhesion (6, 22). Van Loosdrecht et al. (25) reported that cell-water contact angles vary between 21 and 70° without any relationship to a gram-positive or gram-negative cell wall type. For the same set of organisms, electrophoretic mobilities ranged from -0.42×10^{-8} m^2 V^{-1} s^{-1} to -3.09×10^{-8} m^2 V^{-1} s^{-1}. These physicochemical factors reflect the chemical composition of the bacterial cell wall. There exists great diversity in the chemical compositions among different classes of bacteria. Hence, it is difficult to deduce general relationships between the cell wall chemistry and the physicochemical and adhesive properties. However, it should be possible to determine such a relationship for a taxonomically related group of microbial species, such as the coryneform bacteria, whose cell wall composition varies predominantly in the nature and amount of only a few types of chemical units. Recently, the cell wall compositions of bacteria from the genera *Corynebacterium*, *Rhodococcus*, *Gordona*, *Mycobacterium*, *Arthrobacter*, and a nonidentified coryneform strain with a type B peptidoglycan (sensu Schleifer and Kandler [19]) have been characterized and were found to vary, among other components, in the presence and chain length of mycolic acids (3). These compounds are α-branched β-hydroxylated long-chain fatty acids synthesized by the *Corynebacterium-Mycobacterium-Nocardia* group and are covalently bound to an arabinogalactan polymer, which, in turn, is covalently bound to peptidoglycan (14). Chain length, in terms of overall number of carbon atoms, ranges from 22 to 36 in the genus *Corynebacterium* to 60 to 90 in the genus *Mycobacterium* (21). Mycolic acids are a major constituent of the cell wall of *Corynebacterium-Mycobacterium-Nocardia* group bacteria (24) and presumably render the cell surface hydrophobic (9, 23).

The objectives of this study were (i) to confirm the relationship between the properties of cell surfaces of coryneform bacteria (hydrophobicity and electrokinetic potential) and chemical cell wall composition and (ii) to determine the effect of these physicochemical properties on adhesion on defined surfaces. The assessment of these relationships extends understanding of the molecular basis of adhesion and may help select coryneform strains for biotechnological or environmental applications requiring a specific adhesive behavior. Some of the data have been published previously as a symposium abstract (4).

Origins and classification of strains. Detailed description of the chemotaxonomic markers of most of the isolated strains has been published (3). In Table 1, the laboratory designations of the strains are referred to by the numbers used in this paper. *Rhodococcus* sp. strain C125 (formerly *Corynebacterium* sp. strain 125) and *Rhodococcus erythropolis* A177 (formerly *Arthrobacter* sp. strain 177) (20) were obtained from the culture collection of the Department of Microbiology, Agricultural University of Wageningen, Wageningen, The Netherlands. *Mycobacterium fortuitum* CG-2 (16) was kindly provided by M. S. Salkinoja-Salonen, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland.

Cultivation. Bacteria were grown in shake cultures of 500 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 30°C. The medium described by Rijnaarts et al. (17) was used for *Rhodococcus* sp. strain C125 and *R. erythropolis* A177. Cells were harvested by centrifugation and washed twice in 500 ml of phosphate-buffered saline (PBS) with an ionic strength of 0.01 M (containing 8.44 mM NaCl, 0.21 mM KH_2PO_4, and 0.68 mM K_2HPO_4 in deionized water) and a pH of 7.2. Concentrated cell suspensions were obtained by resuspension in 5 ml of PBS (17).

Mycolic acids. Silica gel thin-layer chromatography of whole-cell methanolysates was used to detect and classify mycolic acid methyl esters on the basis of their chain length (3). In addition, for most strains the exact overall number of carbon atoms in mycolic acids was determined by high-temperature gas chromatography of trimethylsilylated (*N*-methyl-*N*-trimethylsilylheptafluorobutyramide [catalog no.

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TABLE 1. Presence and chain length of mycolic acids for the coryneform bacteria isolated and selected reference strains

Strain	Designation no.	No. of carbon atoms in mycolic acids[a]
Arthrobacter "nicotianae" group	1	
DSM 6687[b] (=815/2)	1.1	0
1030/1	1.2	0
1029/2	1.3	0
600/3	1.4	0
2000/1	1.5	0
Coryneform bacteria with type B peptidoglycan	2	
1103/2	2.1	0
DSM 6685 (=1038/2)	2.2	0
1109/1	2.3	0
Corynebacterium spp.	3	
2010/1	3.1	32–36
1094/1	3.2	32–36
DSM 6688 (=1106/1)	3.3	32–36
C. pilosum DSM 20521[T]	3.4	28–36
Corynebacterium sp. with tuberculostearic acid	4	
1033/1	4.1	34–40
1068/1	4.2	34–40
1664/1	4.3	34–40
DSM 44016 (=1032/1)	4.4	34–40
Rhodococcus spp.	5	
C125	5.1	44–56
R. erythropolis A177	5.2	34–48
R. erythropolis DSM 43066[T]	5.3	34–48
R. rhodochrous DSM 43241[T]	5.4	38–48
R. globerulus DSM 43954	5.5	34–48
Gordona spp., rough colonies	6	
G. rubropertinctus DSM 43197[T]	6.1	46–62
1775/15	6.2	52–60
1771/14	6.3	52–60
1730/10	6.4	52–60
DSM 44015 (=1610/1b)	6.5	52–60
Gordona sp., smooth colonies	7	
1610/1a	7.1	52–60[c]
1775/15	7.2	52–60[c]
1763/11	7.3	52–60[c]
1779/13	7.4	52–60[c]
Mycobacterium spp.	8	
2068/19	8.1	60–90[c,d]
1888/19	8.2	60–90[c,d]
M. fortuitum CG-2	8.3	60–90[c,d]

[a] Determined by high-temperature gas chromatography except as noted.
[b] Strains with DSM numbers were deposited at the Deutsche Sammlung für Mikroorganismen GmbH, Braunschweig, Germany.
[c] Chain-length determination was performed with thin-layer chromatography.
[d] Mycolic acid methyl esters exhibited a multispot pattern on a thin-layer chromatogram.

70126; Machery und Nagel, Düren, Germany]) mycolic acid methyl esters according to the method of Klatte et al. (12).

Chemical cell wall composition. Strains of the "nicotianae" group of the genus *Arthrobacter* and the coryneform bacteria with a type B peptidoglycan lacked mycolic acids (Table 1).

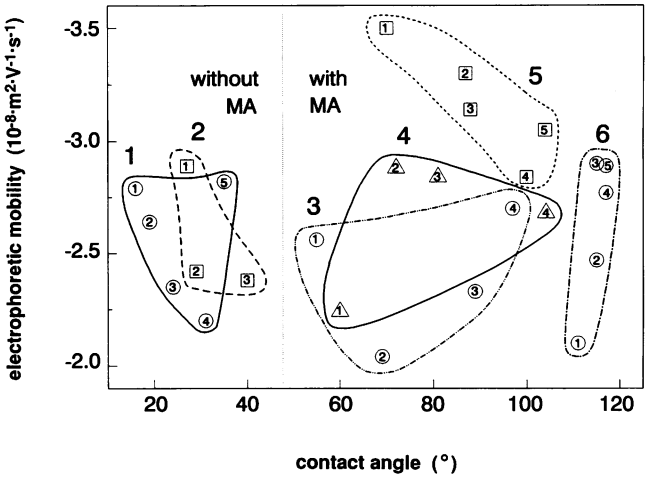

FIG. 1. Clustering of coryneform strains according to their electrophoretic mobilities and contact angles. Strains of the same genus with a similar cell wall composition are circled. For details about the strains associated with the numbers, see Table 1. MA, mycolic acids. Numbers: 1, *Arthrobacter* "nicotianae" group; 2, coryneform bacteria with type B peptidoglycan; 3, *Corynebacterium* spp.; 4, *Corynebacterium* sp. with tuberculostearic acid; 5, *Rhodococcus* spp.; 6, *Gordona* spp., rough colonies.

Strains of *Corynebacterium* spp., *Corynebacterium* sp. with tuberculostearic acid, *Rhodococcus* spp., *Gordona* spp., and *Mycobacterium* spp. possessed mycolic acids with increasing chain lengths (Table 1). The mycolic acid methyl ester multispot patterns of strains 8.1, 8.2, and *M. fortuitum* CG-2 were comparable. Some strains of *Gordona* sp. exhibited both smooth and rough colonies. Subcultivation of a rough colony on complex medium agar plates yielded rough colonies, whereas the smooth variants yielded smooth (97%) as well as rough (3%) colonies.

Physicochemical cell surface properties. Water drop contact angles on air-dried layers of bacteria were measured (25). The contact angle represents the mean of two independent batch cultures. Two bacterial layers were prepared for each batch culture, and subsequently contact angles were measured in triplicate on each layer (standard deviation, ≤4%). Electrophoretic mobilities of the bacterial cells were measured in duplicate by laser-doppler velocimetry at an ionic strength of 0.01 M PBS (26) (standard deviation, ≤10%).

The contact angle and electrophoretic mobility data are shown in Fig. 1. Electrophoretic mobilities varied between -2.0×10^{-8} m^2 V^{-1} s^{-1} and -3.5×10^{-8} m^2 V^{-1} s^{-1} within all groups of strains studied. Contact angles ranged from 16 to 117°. Strains of the same genus with similar cell wall compositions are circled on the figure. The reference strains gave results similar to those of our own isolates.

Hydrophobicity and cell wall composition. The bacterial strains tested could be divided into three distinct clusters in terms of cell surface hydrophobicity: (i) the coryneform bacteria lacking mycolic acids, with contact angles of 16° < Θ < 40°, which is considered to be hydrophilic to moderately hydrophobic; (ii) the coryneform bacteria possessing mycolic acids with an overall number of 28 to 56 carbon atoms, with contact angles of 55 to 103°, which is considered hydrophobic; and (iii) the rough variants of *Gordona* sp. with mycolic acids of 46 to 62 carbon atoms and an extremely high hydrophobicity (111° < Θ < 117°). In contrast to the

extremely hydrophobic rough colony *Gordona* strains, contact angles of 30 to 61° were obtained for the smooth colony variants (strains 7.1 to 7.4 in Table 1). These contact angles came close to the upper range of contact angles measured for the coryneform bacteria without mycolic acids. Mycobacteria (strains 8.1 to 8.3 in Table 1) exhibited contact angles of 85 to 98°. Thus, mycolic acid-possessing bacteria were more hydrophobic than bacteria lacking mycolic acids. Furthermore, there was a tendency towards an increase in contact angle with increasing mycolic acid chain length. However, in some cases, the latter effect was smaller than expected, possibly because of the opposing effect of additional cell surface compounds such as C-mycosides (glycopeptidolipids) (8), trehalose-containing lipooligosaccharides (5), weakly hydrophobic exopolysaccharides (15), proteins (1), and peptidolipids (13).

Electrophoretic mobility and cell wall composition. The high negative surface charge measured for all strains may originate to a great extent from the peptidoglycan which contributes 30 to 70% of the dry weight of gram-positive cells (18, 24). The different peptidoglycan types of the coryneform bacteria under study contain in their peptide side chains or interpeptide bridges amino acids with free carboxyl groups (2, 3). However, for some mycolic acid-possessing bacteria, such carboxyl groups were found to be amidated (11, 24), thus reducing the free net negative charges. Consequently, the amount of peptidoglycan and the degree of amidation of free carboxyl groups have to be determined in order to assess the contribution of peptidoglycan to surface charge. However, other compounds that contain charged groups may also contribute to the observed negative cell surface charge. Phosphate-containing teichoic acids were found to be a dominant accessory polymer in the cell walls of *Arthrobacter* "nicotianae" group strains (7). In cell walls of mycolic acid-possessing bacteria, a phosphate- and carboxyl group-containing lipoarabinomannan has been detected in large quantities (10). In addition, the covalent phosphodiester bridge between the mycolylarabinogalactan and the muramic acid (14) introduces phosphate groups into the cell wall.

Adhesion experiments. Transparent surfaces of PFA-Teflon film (a copolymer of perfluoroalkoxypropylene and polytetrafluoroethylene; Fluorplast, Raamsdonksveer, The Netherlands) and glass (cut from microscope coverslips) were used for adhesion experiments. Glass is hydrophilic (Θ = 12°) and PFA-Teflon is hydrophobic (Θ = 105°) (17). Both surfaces have a negative surface potential of -44 ± 2 mV in 0.01 M PBS as determined by streaming potential measurements (17). The adhesion assays were performed with PBS with an ionic strength of 0.1 M, according to method 1 of Rijnaarts et al. (17). Adhesion data were averaged from triplicate vials, each containing a piece of surface incubated in a suspension of 5×10^8 cells per ml (standard deviation, ≤22%). Adhesion was determined for six strains selected from the different chemotaxonomic groups and was plotted as a function of contact angle (Fig. 2).

For all strains, adhesion on Teflon was better than that on glass, except for the hydrophilic "nicotianae" group strain 1.1 (Θ = 16°), for which adhesion on glass was slightly higher than it was on Teflon. An exceptionally high degree of adhesion on both surfaces was obtained for the coryneform strain 2.2 with a type B peptidoglycan. Rijnaarts et al. (17) showed that the high degree of adhesion of strain 2.2 was caused by a thick layer of polymers protruding several micrometers from the dehydrated cell surface and penetrating diffusion barriers at close proximity to the substratum.

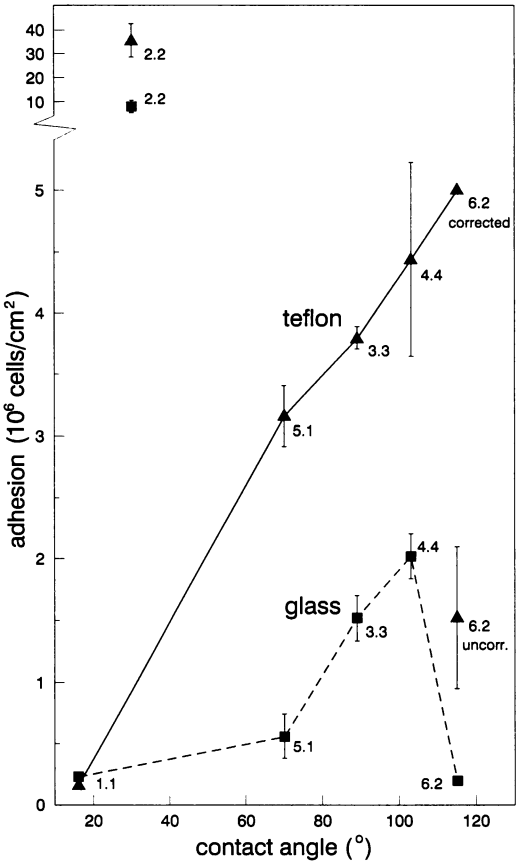

FIG. 2. Adhesion of coryneform strains on Teflon (▲) and glass (■) as a function of cell surface hydrophobicity. For *Gordona* sp. strain 6.2, the value corrected for aggregation is included in the figure. The numbering of strains is according to Table 1. A missing error bar indicates that the standard error was too small for reproduction in the drawing. Uncorr., uncorrected.

The extremely hydrophobic *Gordona* sp. strain 6.2 showed much lower adhesion on both surfaces than the other less hydrophobic coryneform bacteria (strains 3.3, 4.4, and 5.1). This might be due to aggregation of the *Gordona* cells, which caused a dramatic reduction in the concentration of suspended single cells. Therefore, adhesion experiments with this strain were performed at different cell concentrations. The fraction of suspended single cells was determined for each cell concentration, and adhesion is shown in Fig. 3 as a function of suspended single cells. For the initially applied high cell densities of 7.28×10^8/ml and 3.64×10^8/ml, the number of suspended single cells was reduced by aggregation to values of 2.5×10^8/ml and 2.0×10^8/ml, respectively (Fig. 3). For the other cases, suspended single cell concentrations were equal to the total applied cell concentrations. Extrapolation to a suspended single cell concentration of 5×10^8/ml yielded adhesion numbers of 5×10^6/cm^2 for Teflon and 0.3×10^6/cm^2 for glass. This correction procedure led to much higher adhesion values for Teflon (Fig. 2) but not for glass. Thus, adhesion increased in general with increasing cell-water contact angle for both glass and Teflon surfaces, except for the rough *Gordona* sp. strain 6.2 on glass (Fig. 2). This confirms the findings of others (6, 17, 22) that interactions related to hydrophobicity contribute to the adhesion of microorganisms on solid surfaces. The

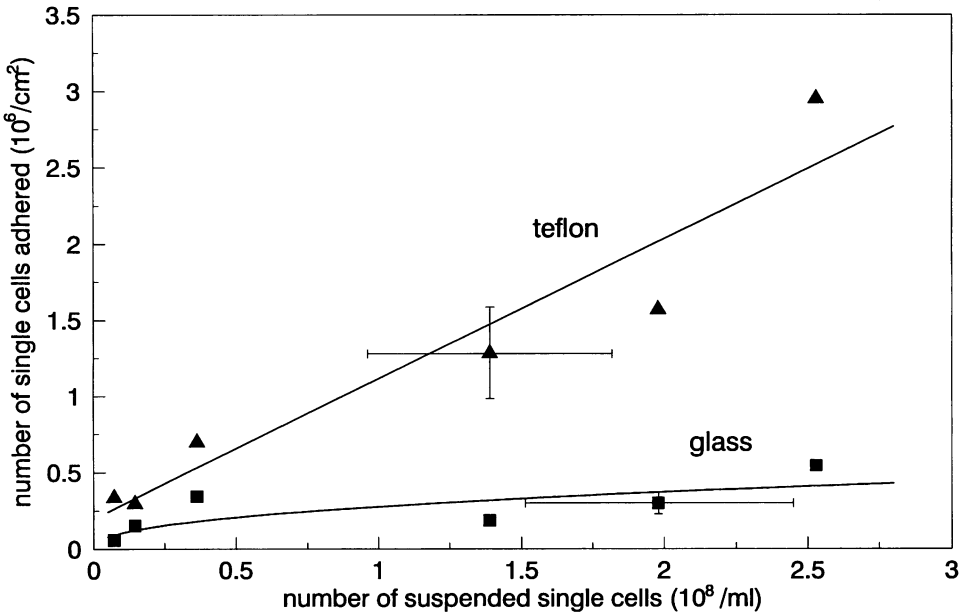

FIG. 3. Adhesion isotherms for single cells of *Gordona* sp. strain 6.2 on Teflon and glass. Total initial suspended cell concentrations were 7.28×10^8/ml and dilutions of this suspension by factors of 2, 5, 20, 50, and 100, respectively. Only single cells were counted, even if a cell suspension contained aggregates as well as single cells, which was the case for the two highest cell concentrations. Adhesion was also determined by counting only single cells. The error bars are given for only one datum point for Teflon and glass, respectively.

deviating behavior of strain 6.2 on glass shows that factors other than hydrophobicity may also affect adhesion.

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