# BACTERIAL ADHESION

CENTRALE LANDBOUWCATALOGUS
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**Promotoren:**

**dr. A.J.B. Zehnder, hoogleraar in de microbiologie**
**dr. J. Lyklema, hoogleraar in de fysische chemie, met bijzondere aandacht voor de grensvlak- en kolloïdchemie**

**Co-promotor:**

**dr. ir. W. Norde, universitair docent bij de vakgroep fysische en kolloïdchemie**

NN08201, 1231

M.C.M. van Loosdrecht

# BACTERIAL ADHESION

Proefschrift
ter verkrijging van de graad van
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NN08201,1231

# STELLINGEN

1. Een bacterie suspensie gedraagt zich, qua stabiliteit, als een kolloidaal systeem.

   Dit proefschrift.

2. Celaanhangsels als fimbriae, pili en fibrillen zijn in fysisch chemisch opzicht uitstékende hechtingsorganellen.

   Dit proefschrift.

3. In tegenstelling tot hun bewering dat substraat limitatie leidt tot "bioflocculatie", tonen Logan en Hunt uitsluitend aan dat convectief substraat transport bij kan dragen aan het totale substraat transport.

   B.E. Logan en J.R. Hunt. 1987. Bioflocculation as a microbial response to substrate limitation. Biotechn. Bioeng. 31:91-101.

4. Bij het onderzoek naar de microbiële afbraak van xenobiotica wordt ten onrechte nauwelijks aandacht geschonken aan de afbraak van natuurlijk voorkomende analoge verbindingen.

5. Studeren is in de eerste plaats een investering in de samenleving.

6. Het gebruik van de term $K_m$ door Bachmann et. al. getuigt niet van een spreekwoordelijke Zwitserse precisie.

   A. Bachmann et. al. 1988. Aerobic biomineralization of alpha-hexachlorocyclohexane in contaminated soil. Appl. Environm. Microbiol. '54:548-554.

7. De conclusie van Fletcher dat gehechte bacteriën een 2 - 5 maal verhoogde metabole activiteit hebben, berust op een verkeerde proefopzet.

   M. Fletcher. 1986. Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces. Appl. Environm. Microbiol. 52:672-676.

8. De conclusie van Busscher et al. dat bacteriële adhesie reversibel is indien de adhesie vrije energie een positieve waarde heeft, is thermodynamisch gezien onjuist.

   H.J. Busscher et al. 1986. Reversibility of adhesion of oral Streptococci to solids. FEMS Microbiol. Lett. 35:303-306.

9. De constatering van Harder dat het centrale aspect van (microbieel) biotechnologisch onderzoek de interactie tussen het (micro) organisme en zijn omgeving is, is in tegenspraak met zijn beeld waarin de microbiële fysiologie centraal staat. Daar dient de microbiële ecologie te staan.

W. Harder. 1987. Microbial physiology, a cornerstone in the development of biotechnology. Proc. 4th ECB. 4:109-120.

10. Omdat er doorgaans geen rekening wordt gehouden met de specifieke geleidbaarheid van bacteriën, zijn vele in de literatuur vermelde waarden voor zeta-potentialen van bacteriën onjuist.

11. Het opdelen van een beperkte ruimtelijke en structurele eenheid in een veelheid van aparte straatjes getuigt van een vergaande vorm van kleinsteedsheid.

12. Positieve discriminatie is een vorm van negatief taalgebruik.

13. Regeren en reguleren worden vaak verward.

Stellingen behorende bij het proefschrift "Bacterial adhesion"

M.C.M. van Loosdrecht Wageningen, 9 september 1988

## CONTENTS

# CHAPTER 1

## INTRODUCTION

### 1.1 GENERAL SCOPE

Adhesion of microorganisms to surfaces and to other microorganisms is ubiquitous in the natural environment as well as in bioreactors. It has already long ago been shown that surfaces can influence microbial activity (12,18,20), and attached organisms are frequently dominant compared to freely suspended cells in many environments ranging from the human digestive system to natural streams (5,8,10,14). The two main questions related to bacterial adhesion are:

1. How do bacteria adhere?
2. What are the advantages of being adhered?

**Mechanisms of adhesion**

Microbial colonization of a solid/liquid interface may occur in the following sequence (Fig. 1):

- **Transport of cells to a surface.**

Bacteria can reach a surface by three different modes:

(i) Diffusive transport. Bacteria exhibit a non-negligible degree of Brownian motion (average displacement 40 μm/h, 15) that can be observed under the microscope. This motion could account for random contacts of small bacteria with interfaces in quiescent conditions or in the viscous sublayer in turbulent flow, but does not significantly contribute to bacterial transport in turbulent flow or of motile cells. Under quiescent conditions sedimentation of bacteria may also contribute to bacterial transport.

(ii) Convective transport. Convective transport is the transport of cells by the liquid flow. Convective transport may be several orders of magnitude faster than diffusive transport. An extensive overview of convective bacterial transport is given by Characklis (4).

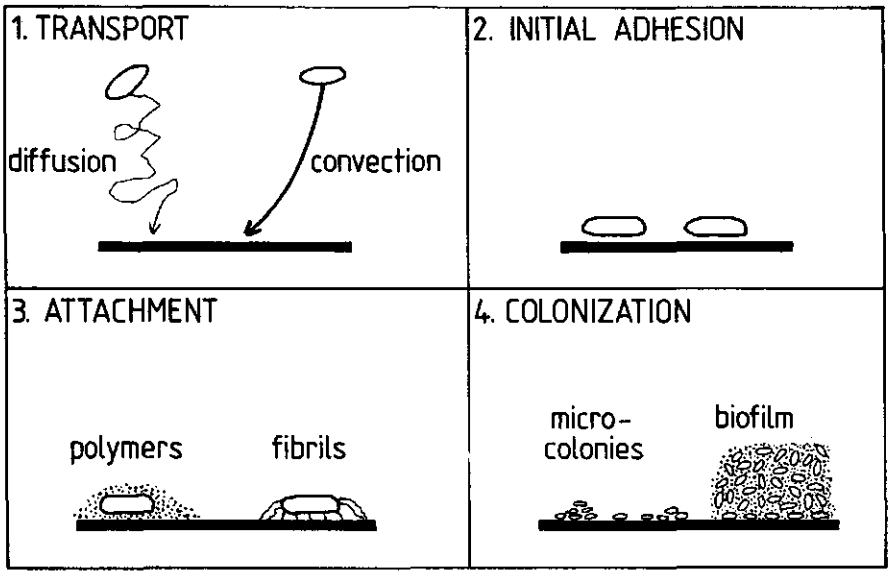


Figure 1 Sequencing steps in the colonization of surfaces by microorganisms.

(iii) Active transport. Once a bacterium is in the vicinity of a surface, it may chemotactically respond to any concentration gradient that may exist in the interfacial region. Such responses do not contribute significantly to the transport under turbulent flow conditions and for non-motile cells.

- **Initial adhesion.** Initial adhesion is mainly a physicochemical process and can be divided into two separate stages, namely reversible and irreversible adhesion. Reversible adhesion may be defined as deposition of bacteria to a surface in such a manner that the bacteria continue to exhibit a two-dimensional Brownian motion and can be removed from the surface by the shearing effects of a water stream or by the bacterium's own mobility. Irreversibly adhering bacteria no longer exhibit Brownian motion and cannot be removed by a moderate shear force. Initial adhesion is further discussed in section 1.2.
- **Firm Attachment.** After the bacterium has been deposited on the solid surface, special cell surface structures (e.g. fibrils or polymers) may form a strong connection between cell and solid surface. Polysaccharides have been shown to be essential for the development of surface films, but not for the initial adhesion of bacteria (2).

- **Surface colonization.** When firmly attached cells start growing and newly formed cells remain attached to each other, micro-colonies or biofilms may develop. In the case of growth of reversible adhering cells, newly formed cells will partly be released into the medium (10).

The last two steps are mainly determined by the type of organism and the environmental conditions, and therefore less generic than the first two steps.

**Advantages of attachment**

The advantages for bacteria to be attached are the following:

- **Preservation of an optimal position.** In systems with high dilution rates bacteria need to attach in order to prevent of being washed-out. Examples are: the digestive tract, UASB reactors, activated sludge systems, the oral cavity and streams.
- **More efficient uptake of substrate.** Microorganisms growing on solid substrates (e.g. cellulose) attach in order to optimize the uptake of exoenzymatic products. Colonization of plant roots by root exudate utilizing bacteria is another example. Suspended cells in a mixed system move with the liquid flow, and substrates can only reach the cells by diffusion. When cells are attached to each other or solid particles an extra substrate transport by convective transport (particles do not move as fast as the liquid flow) can take place.
- **Protection from predation.** Freely suspended cells are easily predated by protozoa or ciliates, whereas attached cells are better protected. This has been fairly well studied in activated sludge (9), and also for soil bacteria (13,110).
- **Physiological advantages.** A few hypotheses predict a physiological advantage for adhered cells (6,7,16). However none of these hypotheses have been experimentally confirmed (see Chapter 7). One of the most used arguments to explain the advantage of attachment is that substrates are accumulated at interfaces, and therefore adhered organisms will be exposed to higher substrate concentrations. However, the net Gibbs energy for (biological) conversions of substrates depends on the chemical potential of the compound, which in the case of adsorption equilibrium is identical for adsorbed and dissolved molecules.

Thus, bacteria cannot profit directly from an increased substrate concentration at the interface.

## 1.2 INITIAL BACTERIAL ADHESION

Bacterial adhesion and coagulation have been studied by scientists from a wide variety of disciplines. Adhesion and/or coagulation are involved in the activity and survival of bacteria in natural habitats, biotechnological processes, medicine, dentistry, waste water engineering, biofouling and in synthrophic and other community interactions between microorganisms and other (micro)organisms. Different approaches to the study of these phenomena have been developed, depending on wether the work was carried out by microbiologists, biotechnologists, dentists, engineers, or colloid chemists.

In order to develop a general model for the understanding and description of initial adhesion it is required to approach adhesion from a fundamental viewpoint. We have approached the complex adhesion phenomenon using some simplifications such as the use of a model surface (sulphated polystyrene disks) and bacteria without surface appendages like fibrils or fimbriae.

Bacteria are, in principle, relatively big colloidal particles. The behavior of colloidal particles is reasonably well described by colloid and surface chemical theories. Therefore, by combining the knowledge and expertise of colloid and interface scientists and microbiologists seems promising to study bacterial adhesion. The literature provides two basic concepts for such a study. The first one is based on the Gibbs energy involved in the destruction and creation of interfaces (1,3). The second concept is based on the DLVO theory for colloidal stability (17).

## 1.3 OUTLINE OF THIS THESIS

A general discussion of physical-chemical theories relevant to particle adhesion is given in Chapter 2. The influence of surface hydrophobicity and electrophoretic mobility of the bacteria on adhesion to a model-surface of sulphated polystyrene is described in the Chapters 3 and 4. In Chapter 5 the results of the Chapters 3 and 4 are combined with the data derived from adhesion isotherms. In this Chapter the applicability of the

concepts mentioned in the previous paragraph is discussed. The DLVO theory was found to be most useful for the description of the initial stage of bacterial adhesion to polystyrene films. In Chapter 6 we discuss how general the DLVO theory and its applications can be used to predict bacterial adhesion. The following surfaces were used: (i) a hydrophilic surface (glass) which is a model surface for more natural surfaces of silicates and other oxides, (ii) protein coated polystyrene as a model for organic coatings on natural surfaces, and (iii) Rhine river sediment. Finally a critical review of the literature on the effects of surfaces to microbial activity, is given in Chapter 7.

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# CHAPTER 2

## THEORETICAL BACKGROUND

### 2.1 INTRODUCTION

Treating bacterial adhesion as a physico-chemical process is complicated by the nature of these particles. Bacteria are far from "ideal" particles. They have no simple geometry or uniform molecular composition. Internal chemical reactions can lead to changes in molecular composition both in the interior and at the surface of bacteria, and molecules and ions may cross the bacterium/water interface. These chemical processes continue also after adhesion. Therefore, the adhered cells are rarely in complete physicochemical equilibrium with their environment.

In the light of above complications we have to ask ourselves in howfar physical chemistry can be used to study microbial adhesion. Probably a good insight can be gained by considering adhesion from a conceptual qualitative level. Applying physico-chemical theories quantitatively, however, must be done with the necessary caution. In this chapter a general background on thermodynamical and colloidal aspects of adhesion will be given.

### 2.2 THERMODYNAMICAL ASPECTS OF ADHESION

To describe bacterial adhesion it has been assumed that the interfaces between solid/liquid (SL) and bacterium/liquid (BL) are replaced by a solid/bacterium (SB) interface (1,3). The underlying assumption of this and other approaches is the change in the interfacial excess Gibbs energy upon adhesion ($\Delta_{adh}G^{\sigma}$, expressed in $J.m^{-2}$), described by:

$$\Delta_{adh} G^{\sigma} = G_{SB}^{\sigma} - G_{SL}^{\sigma} - G_{BL}^{\sigma} \qquad [1]$$

When $\Delta_{adh}G^{\sigma}$ is negative, adhesion is thermodynamically favored, and will proceed spontaneously.

If the molecular composition of the interface, the pressure, and the temperature do not change, eq. [1] may be written, as a balance of interfacial tensions ($\gamma$, expressed in $J.m^{-2}$):

$$\Delta_{adh} G = \gamma_{SB} - \gamma_{SL} - \gamma_{BL} \quad [2]$$

It should be noticed that eq. [1] and [2] only apply if both interacting surfaces make direct contact.

The term "hydrophobicity" is often used in the interpretation of bacterial adhesion. In principle the hydrophobicity of a certain component or surface can be defined as its aversion for water. Hydrophobicity originates from the fact that water-water contacts are thermodynamically more favorable than contacts between two non-polar groups or between a non-polar group and water, i.e. it is a feature of non-polar groups tending to be rejected from an aqueous medium rather than being positively attracted to one another. Generally, the excess Gibbs energy of a surface decreases with increasing hydrophobicity. The hydrophobicity of surfaces can only be characterized semi-quantitatively by assessing the preference for water compared to another phase (e.g. air or hexadecane). Table 1 summarizes methods used to determine the hydrophobicity of bacterial cell walls.

All methods mentioned in Table 1 have their complications, limitations and advantages. Some examples are the following: Prior to measuring contact angles, bacterial cells have to be dried; this may induce changes in the surface structure. In some tests only a division between hydrophilic and hydrophobic

Table 1 Methods used to determine bacterial hydrophobicity

| Method | Reference |
|---|---|
| Contact angle of a drop of liquid on a layer of cells | 1,3,8,15 17,18,19 |
| Partitioning of cells in an aqueous/hydrocarbon two phase system | 4,5,13,15,17 18,19,24 |
| Partioning of cells in an aqueous two phase system | 8,15,28 |
| Salt aggregation | 5,14,19,25 |
| Partitioning of hydrocarbons | 12,16,23 |
| Hydrophobic interaction chromatography | 5,11,14,17,19 |
| Bacterial adhesion as a function of the interfacial energies of the solid and liquid | 1 |
| Direction of spreading of a drop of liquid | 27 |

cells can be obtained (i.e. in the aqueous two-phase and the hydrocarbon /water partition test (15)). These tests are thus qualitative rather than quantitative. Electrostatic interactions may interfere in the salt aggregation test and the aqueous two-phase partition test (2). In the hydrocarbon adsorption test the applied hydrocarbons (i.e. palmitic acid) not only adsorb to the cell surface but also absorb in the cell wall and membrane.

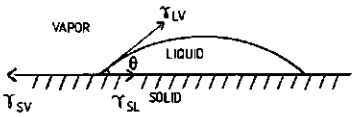


Figure 1
Contact angle measurement.

There is no report systematically evaluating all methods mentioned in Table 1. When different methods are compared, very hydrophobic and very hydrophilic cells behave similarly in all tests, whereas intermediate cells behave differently in different tests (1,5,8,15,17,18,19). Nevertheless, there is consensus on using contact angle measurements as the relatively best method for characterizing bacterial hydrophobicity. The quality of information of this test may be improved by combining it with hydrophobic interaction chromatography or a hydrocarbon/water partitioning test (3,15,19). Because contact angle measurements are regularly used to derive bacterial surface tensions a short discussion on the interpretation of contact angles will be given below.

By measuring the contact angle ($\theta$) of a drop of liquid on a solid surface it is possible to obtain information about the interfacial tensions, by applying Young's equation (see also Fig. 1):

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos\theta \qquad [3]$$

From this equation it follows that, for a given liquid, $\cos\theta$ depends on the difference between $\gamma_{SV}$ and $\gamma_{SL}$. The smaller $\gamma_{SL}$ is (i.e. the more the surface properties of the solid and liquid are alike) the higher $\cos\theta$. The contact angle of water on a surface is therefore large when the surface contains many non-polar (hydrophobic) groups.

The interfacial tension of a solid/vapor interface can be related to the corresponding solid/vacuum interfacial tension ($\gamma_S$):

$$\gamma_{SV} = \gamma_S - \pi_{SV} \qquad [4]$$

The spreading pressure ($\pi_{sv}$ expressed in $J.m^{-2}$) is due to the adsorption of vapor molecules on the solid surface. Determination of the interfacial tensions of the solid/liquid and solid/vapour interface is impossible with Young's equation. A second relation in addition to eq. [3] is needed. Two approaches have been proposed namely the "equation of state" approach (1) and the "geometric mean" approach (3). Both procedures have the same underlying principle, basically a model proposed by Fowkes (7) in which it is assumed that the components contributing to the surface tension are additive. The surface tension is regarded as the sum of a dispersive part ($\gamma_s^d$) due to the London-Van der Waals interaction and a term comprising all other interactions ($\gamma_s^*$), among which dipole-dipole interactions:

$$\gamma_s = \gamma_s^d + \gamma_s^* \qquad [5]$$

For the interaction between two surfaces Fowkes proposed for the dispersion interactions that the interfacial tension will be less than the sum of both surface tensions against vacuum, by an amount approximately equal to twice the geometric mean of both surface tensions:

$$\gamma_{sL}^d = \gamma_s^d + \gamma_L^d - 2(\gamma_s^d \gamma_L^d)^{1/2} \qquad [6]$$

As the interactions between most adjoining phases are not completely dispersive, attempts were made to use the above equations for more polar systems as well. Good (9) critically discussed the correction term and concluded that the term can only be used for polar systems if the interactions in both phases are all of the same type. Otherwise the correction term represents an overestimation. Good introduced a parameter ($\Phi$) with a value between 0 and 1 depending on the nature of the interactions of both phases:

$$\gamma_{sL} = \gamma_s + \gamma_L - 2\Phi(\gamma_s \gamma_L)^{1/2} \qquad [7]$$

$\Phi$ becomes 1 if the interactions in both bulk phases are of the same type.

Neumann et al. (20) found a linear relation between the Good interaction parameter and the interfacial tension:

$$\Phi = -\alpha \gamma_{sL} + \beta \qquad [8]$$

This relation was obtained by (i) determining $\gamma_{sv}$ from a plot of the contact angle of different liquids against $\gamma_{Lv}$; at the point where $\cos\theta$ becomes zero $\gamma_{Lv} = \gamma_{sv}$, (ii) establishing $\gamma_{sL}$ for each

liquid by Young's equation, (iii) calculate $\Phi$ from the obtained values with eq. 7 (so far a negligible spreading pressure is assumed). The empirical constants $\alpha$ and $\beta$ appear to be constants for a broad range of solid/liquid combinations and were estimated to be 0.0075 and 1.0 respectively. From the above Neumann et al. (21) derived the following equation:

$$\gamma_{SL} = \gamma_S + \gamma_L - 2(1-0.0075\,\gamma_{SL})\,(\gamma_S\,\gamma_L)^{1/2} \qquad [9]$$

By combining Young's equation [3] with eq. [6] and [9] we can obtain two equations which may be used to determine $_{sv}$ or $_{sL}$ from contact angle measurements. The first equation, applying to the "geometric mean" approach, is obtained by combining eqs. [3], [4],[5] and [6], and setting $\gamma_{Lv}=\gamma_L$:

$$\cos\theta = \frac{2(\gamma_S^d\gamma_L^d)^{1/2} + 2(\gamma_S^p\gamma_L^p)^{1/2} - \pi_{sv}}{\gamma_L} - 1 \qquad [10]$$

Here, $\gamma_S^d$ can be determined by measuring the contact angle with a completely apolar liquid ($\gamma_L^p=0$, $\pi_{sv}=0$, $\gamma_L=\gamma_L^d$). By using a range of other liquids, and plotting $\gamma_L^p$ versus $\gamma_L(\cos\theta + 1) - 2(\gamma_S^d\,\gamma_L^d)^{1/2}$, $\pi_{sv}$ and $\gamma_S^p$ are obtained. This approach was used by Busscher et al. (3) to determine a quantity that he called the surface tension of cell surfaces. The second equation, applying to the "equation of state" approach, is found by combining eqs. [3] and [9]:

$$\cos\theta = \frac{(0.015\,\gamma_{sv} - 2)(\gamma_{sv}\gamma_{Lv})^{1/2} + \gamma_{Lv}}{\gamma_{Lv}\,[0.015(\gamma_{sv}\gamma_{Lv})^{1/2} - 1]} \qquad [11]$$

With this equation, the solid/liquid and solid/vapor interfacial tension can be calculated from the measurement of one contact angle. Since the denominator can become zero, equation 11 has some mathematical limitations. However, these limitations can be circumvented (20). Computer tables to determine $\gamma_{sv}$ are available (21). The "equation of state" approach has been used by Absolom et al. (1).

In view of the assumptions that have to be made to derive either [10] or [11] and of which it is highly uncertain whether they apply to bacterial adhesion we shall not use $\gamma_S$ but the contact angle as an indication of the hydrophobicity. However, a comparison of the two approaches will be made in chapter 3 on the basis of our data.

## 2.3 COLLOIDAL ASPECTS OF BACTERIAL ADHESION

Bacteria may be considered as living colloidal particles. Usually they have a net negative surface charge. If a particle approaches a surface it interacts with this surface. Derjaguin, London, Verwey and Overbeek (DLVO) have postulated that the total long range interaction (> 1 nm) is a summation of Van der Waals and Coulomb interactions (26). For simplicity we will, at this stage, ignore steric interactions due to individual polymer chains at the surface. Steric interactions are briefly treated in chapter 6. In contrast to the above surface chemical approach the colloid chemical approach describes the interaction between a particle and a surface as a function of the separation distance.

(i) **Van der Waals interaction.**

Due to correlation in the electronic motion, two atoms attract each other if they are at short separation. In this interaction, an instantaneous dipole moment in the one atom induces an instantaneous dipole moment in the other atom. Generally the attraction is strong between atoms having high ionization potentials. Although the dispersion interaction energy between two atoms varies with $h^{-6}$ (h is the distance between the two atoms), for particles the dispersive interaction has a much longer range ($h^{-1}$, for interaction between a flat plate and a sphere) because the total dispersion interaction is the sum of all the individual atom-atom interactions. The strength of this dispersion interaction between two particles at given separation is expressed by the Hamaker constant (10).

Nir (22) showed that in addition to dispersive also (random) dipole-dipole and (random) dipole-induced dipole interactions should be incorporated in the Hamaker constant. These interactions can also be obtained from a summation of the interactions between single atom pairs. Especially for biological interfaces (with many (induced dipoles) this gives a considerable deviation from the classical Hamaker constant. A complete theoretical background on this subject is given by Nir (22).

The Hamaker constants (A) for the interaction between bacteria (denominated 1), $A_{131}$, and for that between bacteria and surfaces (denominated 2), $A_{132}$, across a medium (denominated 3), are

related to the Hamaker constants of the individual components of the system (29) as follows:

$$A_{131} = A_{11} + A_{33} - 2A_{13} \approx (A_{11}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})^2 \qquad [12]$$

and

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23} \approx (A_{11}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})^2(A_{22}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})^2 \qquad [13]$$

From eq. [13] it is obvious that the Hamaker constant for the interaction between surface and bacterium is smaller if $A_{11}$ and $A_{33}$ or $A_{22}$ and $A_{33}$ are more alike. The more hydrophobic a bacterium or surface is, the more its individual Hamaker constant deviates from that of water, and the larger the Hamaker constant for the total interaction will be.

As discussed before, the water contact angle is large for hydrophobic surfaces. Therefore the contact angle may give semi-quantitative information on the value of $A_{131}$ for different solids. As bacteria consist for a great part of water, $A_{131}$ will be relatively small. Calculations, using the Lifshitz theory (22), give values of 2-6 $10^{-21}$ J for the mutual interaction between two lipid vesicles, coated with a mixture of sugar, protein and water, in an aqueous phase.

(ii) **Electrostatic interaction.**

If only the charge on the particles would determine the electrical interaction, it must be expected that two likewise charged particles repel each other according to Coulomb's law; i.e. the energy would be proportional to the reciprocal distance. Because of electroneutrality the charge on colloidal particles is neutralized by a countercharge that is diffusely distributed

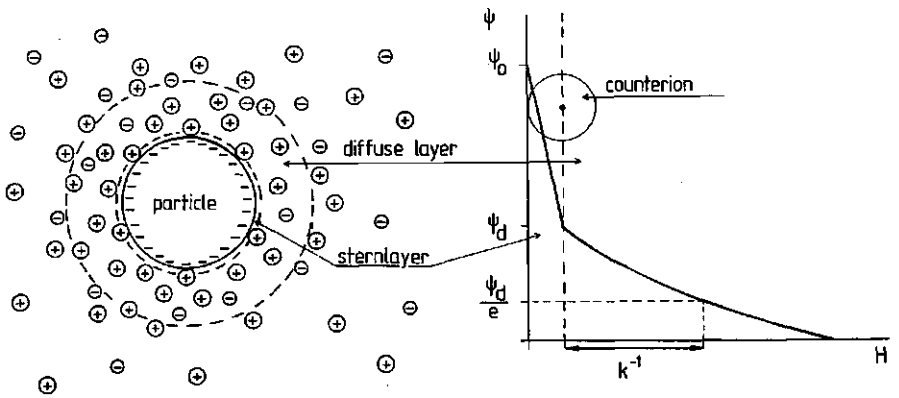


Figure 2 Charge distribution around a colloidal particle.

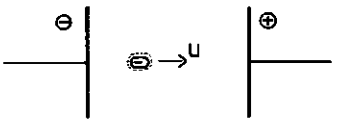


Figure 3
Electrophoretic mobility (u) measurement.

around the particle. The system of a charge on the particle anda countercharge is comparable to a condensator and is therefore called an electrical double layer (Fig. 2). The surface chargeis shielded by the countercharge. As a result the electrical interaction between two particles is smaller than predicted by Coulomb's law. The diffuse layer is compressed by an increase in ionic strength, leading to reduction of the electrostatic interaction at given distance of separation. The mobility of bacteria in an electric field (Fig. 3) is a measure for the electrokinetic potential of the bacteria. This measurement can thus be used to determined the extent of electrokinetic interactions. The electrophoretic mobility measurement of bacteria has been discussed by Einolf et al. (6).

**(iii) Total interaction.**

Fig. 4 shows the electrostatic ($G_E$), Van der Waals ($G_A$) and total interaction energy ($G_{tot}$) as a function of separation (H) for two likewise charged particles, for different ionic strengths. At low ionic strength (a) $G_{tot}(H)$ has a positive maximum that constitutes a barrier for adhesion in the primary minimum. The maximum in $G_{tot}(H)$ is suppressed by increasing ionic strength, due to a reduction of $G_E$. At certain intermediate values of the

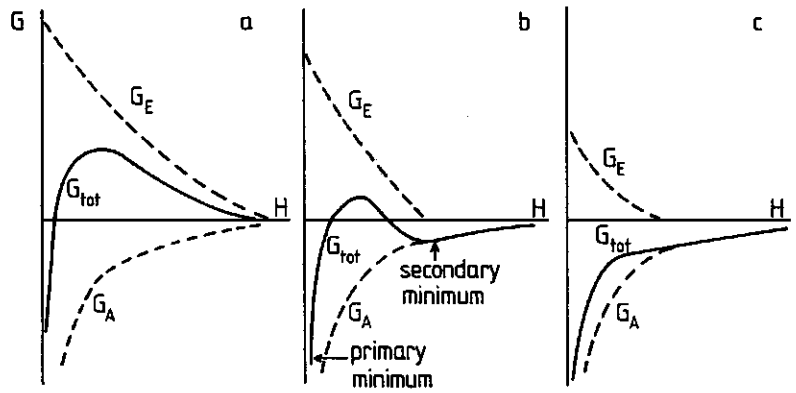


Figure 4 Gibbs energy of interaction between two bodies having the same charge sign. (a) low, (b) intermediate, and (c) high ionic strength.

ionic strength (b) the maximum is so low that a fraction of the particles may contain sufficient thermal energy to pass the barrier (i.e. slow adhesion takes place). At higher ionic strength (c), when $G_{tot}(H) \leq 0$, all particles can reach the primary minimum. This results in a strong, irreversible binding.

At a somewhat larger separation another, a more shallow, minimum in $G_{tot}(H)$ exists: the so-called secondary minimum. It is most pronounced at intermediate ionic strengths and is deeper for systems having a larger Hamaker constant and for relatively large particles, like microbial cells. If the secondary minimum does not attain large values particles in this minimum are reversibly attached. It will almost be needless to say that with opposite charges on the interacting particles $G_{el}$, and thus $G_{tot}$, is negative at all separations, which results in primary minimum adhesion.

At short separation, say $H < 1$ nm, short range interactions (e.g. hydrogen bonding, ion pair formation, etc.) are effective. They determine the strength of adhesion in the primary minimum. The DLVO theory is only able to predict whether primary minimum adhesion occurs, but the depth of this minimum cannot be predicted very well because short range interactions are not incorporated in this theory, and just those determine the position of the minimum and, hence, its depth.

## 2.4 CONCLUSION

A consideration of long range (DLVO) interaction between micro-organisms and surfaces can provide a useful first approach to explain initial processes in bacterial adhesion. In the forthcoming chapters we will deal with the role of these interactions in bacterial adhesion. Specificity of bacterial adhesion to a particular surface cannot, however, be explained in terms of long range interactions.

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# CHAPTER 3

## THE ROLE OF BACTERIAL CELL WALL HYDROPHOBICITY IN ADHESION.


### ABSTRACT

In this study the adhesion of bacteria differing in surface hydrophobicity was investigated. The cell wall hydrophobicity was measured as the contact angle of water on a bacterial layer, collected on a microfilter. the contact angles ranged from 15° to 70°. This method was compared with procedures based upon adhesion to hexadecane, and with the partition of cells in a polyethyleneglycol/dextran two phase system. The results obtained with these three methods agreed reasonably well. The adhesion of sixteen bacterial strains was measured on sulphated polystyrene as the solid phase. These experiments showed that hydrophobic cells adhered in a greater extent than hydrophilic cells. the extent of adhesion correlated well with the measured contact angles (linear regression coefficient 0.8).


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## 3.1 INTRODUCTION

Since the beginning of this century different reports were published which suggest that solid/liquid interfaces can have a considerable effect on bacterial physiology. As early as 1913 Söhngen (22) has shown that inorganic colloids may influence a variety of microbial processes in the soil (e.g. nitrogen fixation, denitrification, etc.). In the fourties Zobell (25) inferred that solid surfaces are beneficial to bacteria in dilute nutrient solutions. this view was supported by Stotzky and Rem (23) who found a stimulating effect of montmorillonite clay on the activity of a number of bacteria. In recent years, these and other observations led to more detailed investigations concerning the influence of solid surfaces on microbial activity (5). Despite the recognition that solid surfaces may influence microbial activities, a good explanation for the observed phenomena is still lacking. Even the adhesion behavior of bacteria is not yet fully understood.

A few authors have described bacterial adhesion in terms of surface Gibbs energy (1,3,6,7). the surface Gibbs energy was calculated from the contact angle of a drop of water or another liquid on a given surface or on a closed layer of bacteria. The contact angle ($\theta$) of a drop of liquid (L) on a solid surface (S) is a function of the three different surface Gibbs energies involved and may be quantified in terms of the three surface tensions ( $\gamma$ ,expressed in $N.m^{-1}$) through Young's equation:

$$\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL} \qquad [1]$$

Experimentally, it is not possible to determine the surface tensions of the solid/liquid ( $\gamma_{SL}$) and the solid/vapor ($\gamma_{SV}$) interface independently. therefore a second relation in addition to eq. [1] is needed. Fowkes (8) proposed a (non-thermodynamic) relation in which the interfacial tension is the geometric mean of the surface tension of the two interacting phases. Two approaches based on this assumption are usually used to estimate the solid surface tension, namely the "geometric mean" (3) and the "equation of state" (1). In the former approach it is assumed that the total surface tension is the sum of a dispersive part (due to the London-van der Waals interactions) and a term

comprising all other interactions (e.g. dipole-dipole, hydrogen bonding etc.). For theoretical backgrounds of both approaches the interested reader should refer to Fowkes (8), good (10), and Neumann et al. (13). As there is some discussion regarding the relative quality of both approaches (3,21) we will compare them to show that the practical results are comparable.

From the above it becomes evident that the solid surface Gibbs energy as a thermodynamic quantity cannot be calculated from the contact angle but can only be estimated by making some non-thermodynamic assumptions. The contact angle, however, is a relative measure of the hydrophobicity of the surface which in most cases shows a correlation with the surface Gibbs energy (the surface Gibbs energy decreases with increasing hydrophobicity). Nevertheless, the data in this paper are solely interpreted in terms of hydrophobicity (because this is what is measured by contact angles) and the terms surface Gibbs energy or surface tension will only be used when referring to other authors who consistently use this term in their publications.

In addition to the contact angle method, the hydrophobicity of bacteria can also be determined by partitioning bacteria between two aqueous phases (9) or by quantifying the number of bacteria adhering to droplets of organic solvents (17). The former method is based on the partitioning of bacterial cells between a polyethyleneglycol (Peg) and a dextran (Dex) phase. A simple calculation shows that theoretically the majority of the cells will move to one phase depending on their surface Gibbs energy (Other interactions e.g. steric or electrical are neglected). The partition of particles over two phases is defined by the following equation:

$$\ln K = {}^{\Delta}{}_{part}G \,/\, RT \qquad [2]$$

where K is the partition coefficient, ${}^{\Delta}{}_{part}G$ the difference in surface Gibbs energy of the particle surfaces between the two phases (expressed in $J.mol^{-1}$), and R and T have their usual meaning. The quantity ${}^{\Delta}{}_{part}G$ can be computed by multiplying the total surface area of one mole of bacteria (A) with the difference in molar surface Gibbs energies of the bacteria in the two different phases. Since the surface area of 1 mole of cells is approx. $2.10^{12}$ $m^2$, already for very small differences in the

surface Gibbs energy the partition coefficient will reach extreme values. As a result all the cells will move to either one of the phases rather than distribute more or less evenly over the two phases. Only in one special case all cells will move to the interface. This occurs if the product of the contact area of the bacterium, located in the interface with Peg ($A_{Peg}$) and the difference of the surface Gibbs energies between cell-Peg ($G_{BPeg}$) and cell-dextran ($G_{BDex}$) is smaller than the product of the area occupied by the bacterium in the Peg/dextran interface ($A_I$) and the surface Gibbs energy of Peg/dextran ($G_{PegDex}$):

$$A_{Peg}(G_{BPeg} - G_{BDex}) < A_I G_{PegDex} \qquad [3]$$

Using equation 3 and a $G_{PegDex}$ of 0,06 $mJ.m^{-2}$ (20) it can be calculated that bacteria move to the interface if the difference in surface Gibbs energy of the bacteria in the two phases is smaller than 0.036 $mJ.m^{-1}$. This condition is satisfied when the bacterial surface Gibbs energy is approximately 58-62 $mJ.m^{-2}$, ($G_{Dex}$= 60 $mJ.m^{-2}$, $G_{Peg}$= 59 $mJ.m^{-2}$), which would correspond with a contact angle of 34° to 41° (14). the finding that a specific bacterial population concentrates at the interface can be used to check the quantitative validity of contact angle measurements.

In this chapter data are presented on the hydrophobicity of 23 different bacterial strains and this hydrophobicity is related to the adhesion of the cells to negatively charged polystyrene. In addition, the mentioned methods to measure hydrophobicity are compared and their applicability critically evaluated.

## 3.2 MATERIALS AND METHODS

**Preparation of bacterial suspension**

All strains investigated in this study were obtained from the culture collection of the Department of Microbiology, Agricultural University, Wageningen. The following strains were used: *Acinetobacter* 210A, *Agrobacterium radiobacter*, *Alcaligenes* sp. (A157), *Arthrobacter globiformis* (Ac8), *Arthrobacter simplex* (A20), *Arthrobacter* sp (A177), *Arthrobacter* sp (A127), *Azotobacter vinelandii* (A59), *Bacillus licheniformis* (B9), *Micrococcus luteus* (M59), *Mycobacterium phlei* (M9), *Pseudomonas fluorescens* (P9), *Pseudomonas aeruginosa* (P8), *Pseudomonas putida* (P11), *Pseudomonas* 26-3, *Pseudomonas* sp (P52), *Pseudo-*

*monas* sp (P80), *Rhizobium leguminosarum* (R6), *Rhodopseudomonas palustris*, and *Thiobacillus versutus* (ATCC 25364).

Bacteria were grown in mineral salt medium containing (per litre of distilled water): 1.93 g $KH_2PO_4$; 7.93 g $K_2HPO_4$; 0,75 g $NH_4Cl$; 0.05 g $MgSO_4$; and 1 ml trace element solution (24). Ethanol (4 ml/l) was used as the sole carbon and energy source because it has minimal interactions with surfaces (it is uncharged and has a low octanol/water coefficient). Strains showing no growth on ethanol (*A. vinelandii, E. coli, M. luteus*) were grown on nutrient broth. The incubation temperature was 30°C.

After 40 h of incubation bacteria were harvested by centrifugation and washed twice in 0.1 M phosphate buffered saline (PBS) containing 0.29 g/l $KH_2PO_4$; 1.19 g/l $K_2HOP_4$; 4.93 g/l NaCl. For adhesion experiments cells were resuspended in PBS to a final concentration of 1-3.$10^8$ cells/ml. Before using the cell suspensions, they were filtered through an 8 μm micropore filter to remove large cell agglomerates.

**Measurement of bacterial hydrophobicity**

**a) Contact angle measurement**

Bacterial surfaces for measuring contact angles were prepared by collecting bacterial cells on a 0.45 μm micropore filter. The filters with a continuous bacterial layer were mounted on glass slides and dried in an desiccator for 0.5 to 3 h. Then the contact angle of an 0.1 M NaCl solution with the bacterial surface was measured. No change in contact angle occurred between 0.5 and 3 h. This is in accordance with findings of Absolom et al. (1) and Busscher et al. (3). Incidentally, a method developed by Absolom (1) was used in which a bacterial film was prepared on agar instead of a micropore filter. Contact angles were measured directly with the aid of a microscope with a goniometric eyepiece (Krüss GmbH, Hamburg). Each reported contact angle is the mean of at least six independent measurements.

**b) Partition of cells in two phase systems**

Relative measurements of the bacterial hydrophobicity developed by Rosenberg (17) and Gerson (9) were compared to contact angle measurements. The first method is based on adhesion of cells to hexadecane droplets. the second method is based on the partition of cells in a two phase system of an 8% dextran (Pharmacia T500) and a 6% polyethyleneglycol (Merck 6000) solution in water. Surface tension of the polyethyleneglycol and dextran solutions were measured with a Wilhelmy plate tensiometer.

**Preparation of polystyrene disks**

Negatively charged polystyrene latex (containing $-OSO^{3-}$ groups) was prepared according to the prescription of Goodwin et al (11); as the initiator 3 mM $K_2S_2O_8$ was used. the obtained latex was dialyzed, freezedried, and subsequently dissolved in toluene (7%, w/w). Thirty ml of this solution was poured into a glass petri-dish (Ø 12 cm) with a flat bottom, which was mounted horizontally. The toluene was allowed to evaporate slowly over a period of three days. The obtained polystyrene film was cut into disks (Ø 1 cm), which were stored dust-free. For adhesion experiments the air-dried side of the disks was used (4). This side has a contact angle for water of 70°. the amount of charged groups per surface area could not be established. From the electrophoretic mobility of the original latex particles (- 7.8 $10^{-8}$ m/Vs in 0.01 M PBS) it can be inferred that the polystyrene disks have a considerable negative surface potential.

**Adhesion experiments**

Freshly prepared bacterial cell suspensions were incubated together with polystyrene disks, on a rotary shaker at 25°C. After incubation for half an hour, the disks were taken from the suspension and rinsed gently for thirty seconds in 0.1 M PBS to remove non attached cells. The rinsing was performed by moving the disks slowly through the water, to prevent detachment of cells due to shear forces. A possible transfer of the cells from the polystyrene surface to the air/water interface during the washing procedure could not occur because a drop of liquid always remained on the disk during the washing procedure. The number of cells adhering to the surface were counted under a light microscope with a calibrated eyepiece. The surface coverage was calculated by multiplying the number of cells per square meter by the cross section area of the cell.

## 3.3 RESULTS

In a first attempt we tried to measure contact angles of bacterial deposits according to the method described by Absolom et al. (1). Although the procedure was followed closely, we were not able to obtain reasonable contact angles. The bacteria were washed away from the agar by the drop of water placed on them. The measured contact angles (approx. 17°) did not differ very much for the tested bacteria, and resembled closely the contact angle of clean agar. Other authors (Busscher, personal

communication) had the same experience. Measurement of contact angles on bacteria collected on micropore filters gave more meaningful results.

The results of the contact angle measurements are summarized in Table 1. The scatter in the contact angle was relatively small (± 1°) indicating that the bacterial film surface was rather homogeneous. The contact angles for different strains can deviate strongly from one to another, even within the same genus. No direct correlation between contact angle for gram-positive or gram-negative cell walls was observed. To test the agreement between the "geometric mean" (3) approach and the "equation of state" (1) approach for the estimation of the surface tension ($\gamma_{sv}$) or its dispersive part ($\gamma^{d}$), the contact angle of α-bromonaphtalene (a completely apolar liquid) and an 0.1 M NaCl solution on a bacterial layer were measured. As can be seen in Table 2 both approaches gave almost identical results. This is not surprising, since the "geometric mean" and the "equation of state" approach have essentially the same theoretical basis (model proposed by Fowkes, 8).

Table 1 Contact angles for different bacteria.

| | Strain | Contact angle (°) |
|---|---|---|
| 1 | *Pseudomonas fluorescens* | 21.5 ± 1.5 |
| 2 | *Pseudomonas aeruginosa* | 25.7 ± 0.9 |
| 3 | *Pseudomonas putida* | 38.5 ± 1.0 |
| 4 | *Pseudomonas* 26-3 | 20.1 ± 0.8 |
| 5 | *Pseudomonas* 52 | 19.0 ± 1.0 |
| 6 | *Pseudomonas* 8 | 29.5 ± 0.5 |
| 7 | *Escherichia coli* NCTC 9002 | 15.7 ± 1.2 |
| 8 | *Escherichia coli* K 12 | 24.7 ± 0.4 |
| 9 | *Arthrobacter globiformis* | 23.1 ± 0.7 |
| 10 | *Arthrobacter simplex* | 37.0 ± 0.9 |
| 11 | *Arthrobacter* 177 | 60.0 ± 1.5 |
| 12 | *Arthrobacter* 127 | 38.0 ± 1.3 |
| 13 | *Micrococcus luteus* | 44.7 ± 0.9 |
| 14 | *Acinetobacter* 210A | 32.6 ± 0.5 |
| 15 | *Thiobacillus versutus* | 26.8 ± 0.8 |
| 16 | *Alcaligenes* 175 | 24.4 ± 0.5 |
| 17 | *Rhodopseudomonas palustris* | 34.3 ± 0.5 |
| 18 | *Agrobacterium radiobacter* | 44.1 ± 0.5 |
| 19 | *Bacillus licheniformis* | 32.6 ± 0.5 |
| 20 | *Corynebacter* 125 | 70.0 ± 3.0 |
| 21 | *Azotobacter vinelandii* | 43.8 ± 0.5 |
| 22 | *Rhizobium leguminosarum* | 31.0 ± 1.0 |
| 23 | *Mycobacter phlei* | 70.0 ± 5.0 |

To examine to what extent the preparation procedure of a bacterial layer for contact angle measurements influences the cell surface hydrophobicity, a comparison was made between the contact angle measurement and the behavior of bacteria in two different two phase systems. The experimental set-up of both measurements is shown in Fig. 1. From the relation between the contact angle measurements and the adhesion to hexadecane droplets (Fig. 2) we concluded that bacteria with a contact angle below 30° do not adhere to the hydrocarbon phase. Above this critical contact angle the adhesion increased concomitantly with the contact angle. Although important deviations occur, the general trend in the partition of bacteria in the Peg/Dextran system follows approximately the contact angle measurements (Fig. 3). Three out of four bacterial strains expected to concentrate at the interface actually did so. The contact angle measurements have also a predictive value for the adherence of bacteria to negatively charged polystyrene (Fig. 4). Correlation between coverage of a surface and contact angle measurements on these surfaces has also been reported elsewhere (1,3,6,16,18). A good correlation between bacterial adhesion and the hexadecane test has already been reported earlier (17).

Table 2 Comparison of calculated surface Gibbs energies by the equation of state and the geometric mean approach[a)]

| Strain | Contact angle (°) | | Equation of state approach ($mJ.m^{-2}$) | | Geometric mean approach ($mJ.m^{-2}$) | |
|---|---|---|---|---|---|---|
| | α-Bromo-naphtalene | Water | $\gamma^d$ | Water $\gamma_{sv}$ | $\gamma^d$ | Water $\gamma_{sv}$ |
| *Pseudomonas* sp. strain 26-3 | 25 | 20 | 41 | 68 | 40 | 70 |
| *Arthrobacter globiformis* | 20 | 23 | 42 | 67 | 42 | 72 |
| *Arthrobacter* sp. strain 177 | 37 | 60 | 36 | 47 | 36 | 48 |
| *Micrococcus luteus* | 31 | 44 | 38 | 56 | 39 | 60 |
| *Veillonella alcalescens* | 57 | 20 | 28 | 68 | 27 | 68 |
| *Streptococcus sanguis* | 41 | 42 | 34 | 57 | 34 | 59 |
| *Streptococcus salivarius* | 44 | 26 | 33 | 65 | 33 | 67 |
| *Streptococcus mitior* | 31 | 55 | 38 | 49 | 38 | 53 |

[a)] The data for *V. alcalescens, S. sanguis, S. salivarius,* and *S. mitior* were taken from Busscher et al. (3) and are used here as additional data to show the agreement between the equation of state and geometric approaches.

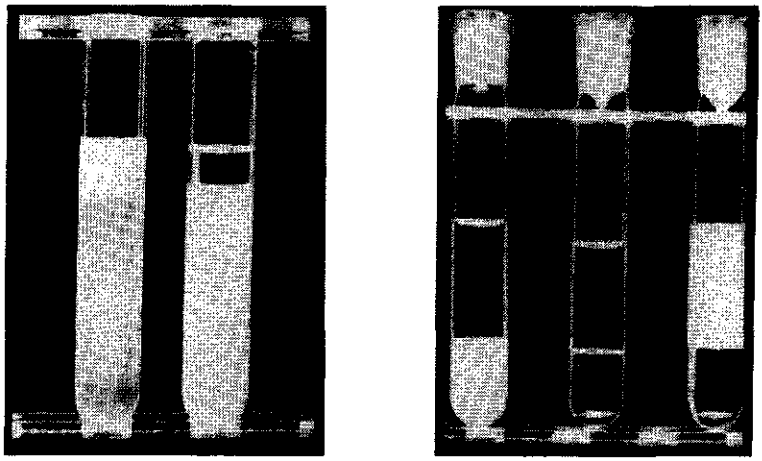

Figure 1 Experimental result of two hydrophobicity tests. Left photograph: Adhesion of *Arthrobacter* sp. strain 177 (left) and *Pseudomonas* sp. strain 26-3 (right) to hexadecane. Right photograph: Partitioning of *Arthrobacter* strain 177 (left), *Arthrobacter* strain 127 (center), and *Pseudomonas* sp. strain 26-3 (right) in a PEG-DEX two-phase system.

### 3.4 DISCUSSION

Measurement of bacterial hydrophobicity can be of importance in many research areas e.g. biofouling, oral microbiology (3), phagocytosis (15), soil microbiology etc. Therefore, a good measure for bacterial hydrophobicity is needed. The use of a broad range of various tests (18) makes it difficult to compare the outcome of the different studies. It may be worth while to initiate some test series in different laboratories with a few reference strains. A thorough evaluation of the results may lead to a generally accepted standard hydrophobicity test. In the following part we will evaluate the three methods used to measure surface hydrophobicity and discuss the possible practical problems and shortcomings.

The measurement of contact angles of an aqueous 0.1 M NaCl solution with a layer of bacteria gave reproducible results, despite the fact that the bacterial layer had to be dried slightly before measurements could be performed. Contact angles correlated relatively well ($r^2$=0.8) with the adhesion of bacteria to negatively charged polystyrene (Fig. 4). From these findings and the data reported in literature (1,3) it can be concluded that contact angles are very useful to estimate the hydrophobicity of the cell surface of a given organism and consequently provides an important factor to predict its adhesion to various surfaces.

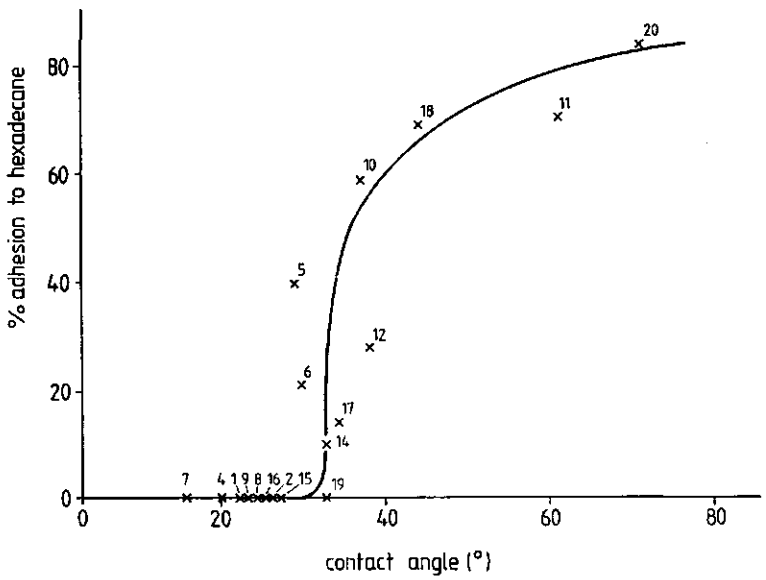


Figure 2 Relationship between contact angle and adhesion of bacteria to hexadecane. Numbers refer to the numbering of the starins in Table 1.

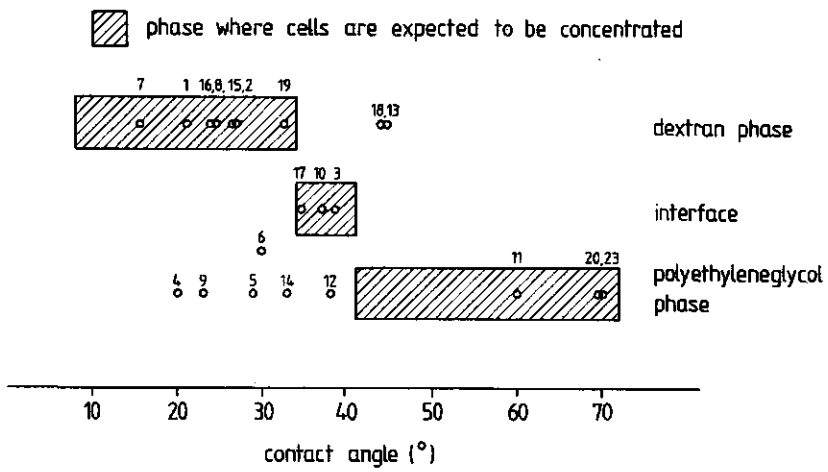


Figure 3 Relationship between contact angle and partitioning of cells in a Polyethyleneglycol-Dextran two-phase system. Numbers refer to the numbering of the strains in table 1.

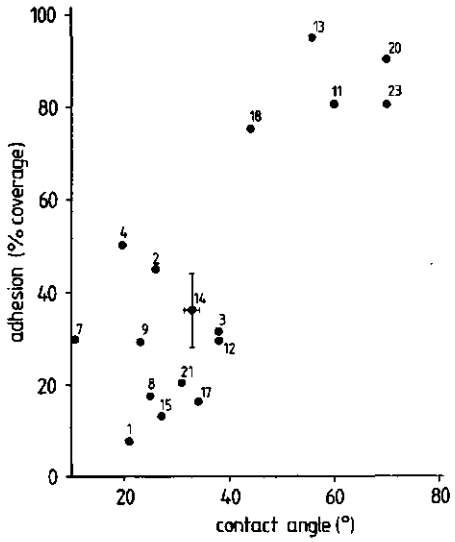


Figure 4 Relationship between bacterial hydrophobicity as determined by contact angle measurements and adhesion to negatively charged polystyrene. Numbers refer to the numbering of the different bacteria in Table 1. Bars indicate the average standard deviation in the measurements.

Analyses of such data in terms of individual surface Gibbs energies or surface tensions, as done in the "equation of state" and "geometric mean" approaches involves a non-thermodynamic assumption and should therefore be avoided; the more so as the use of surface Gibbs energies to calculate the adhesion energy (1,3) is restricted to those cases were bacteria and solid make direct contact whereby the original phase boundaries are replaced by new ones. In the experiments reported here, cells may be adhered at a certain distance from the solid surface at the so-called secondary minimum of the DLVO-theory (12,19). In that case no new boundaries are formed and a balance of surface Gibbs energies will overestimate the adhesion Gibbs energy.

In the hexadecane test the removal of cells from the aqueous suspension depends on their adhesion to the hydrocarbon phase. Thus, this method is very sensitive to the amount of surface area created during mixing of the two liquid phases. This surface area in return is dependent on size and amount of hexadecane droplets by mixing conditions, like temperature, type of mixing vessel, etc.. Since this method is not standardized, data obtained

in different laboratories might show some deviations. A second problem consists in the formation of small hexadecane droplets stabilized by bacteria ("Pickering"-stabilization) which do not leave the water phase. This emulsion may affect the measurement because the adhesion is measured as a decrease in extinction. However, this can be circumvented by microscopically counting the bacteria in the water phase. Several bacterial strains showed a tendency to form stable emulsions, especially *Micrococcus luteus* showed this behavior. Besides these technical problems the quantification of hydrophobicity may be affected by the extraction of cell surface components by hexadecane. A further disadvantage of the hexadecane method is its insensitivity towards differences in hydrophobicity in rather hydrophilic bacteria (Fig. 2).

the partition of cells in the two phase Peg/Dextran system is very sensitive for details in surface structures because $\Delta_{part}G$ is determined by a delicate balance of surface Gibbs energies and steric, electrical and various other interactions, which are not all determined in the contact angle measurement. On the basis of the contact angle measurements three out of four bacteria, expected to concentrate at the interface were actually found there. Not all bacteria did behave as expected from the contact angle measurement, which indicates interactions other than hydrophobicity may also play a role in the partitioning of bacteria. A practical problem is that both phases are relatively viscous, which means that the mixture needs to be shaken very intensively, and the time to allow phase separation must be long (24 h). If the two conditions are not entirely fulfilled an incorrect partition equilibrium will be obtained. Also in this case, microscopy can help to determine quickly to which phase the bacteria have been transferred without having to wait for full separation.

In conclusion we can say: Contact angles are a good measure for bacterial hydrophobicity and have a predictive value for adhesion. Because of the shortcomings of the existing models to generate absolute values for the hydrophobicity of bacterial cells, interpretation of such data in terms of bacterial surface Gibbs energy is suspicious. Because of the importance of bacterial

adhesion in a great variety of technologies and natural processes, there is an urgent need to come to one generally accepted method for the measurement of cell hydrophobicity. Based on the data reported in literature and our own findings we propose to use the water contact angle measurement to quantify cell hydrophobicity.

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# CHAPTER 4

## ELECTROPHORETIC MOBILITY AND HYDROPHOBICITY AS A MEASURE TO PREDICT THE INITIAL STEPS OF BACTERIAL ADHESION


### ABSTRACT

The relation between physico-chemical surface parameters and adhesion of bacterial cells to negatively charged polystyrene was studied. The cell surface hydrophobicity and electrokinetic potential were determined by contact angle measurement and electrophoresis, respectively. Both parameters influence bacterial adhesion. The effect of the electrokinetic potential increases with decreasing hydrophobicity. Cell surface characteristics determining adhesion are influenced by growth conditions. At high growth rates bacterial cells tend to become more hydrophobic. This fact can be of ecological significance by controlling the spreading of bacteria throughout the environment.


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## 4.1 INTRODUCTION

Bacterial adhesion has been interpreted in terms of hydrophobicity or surface Gibbs energy (1,2,12). Although some authors have indicated an influence of electrical charges of bacteria and solid surfaces on adhesion (7,8,10,15), the influence of electrostatic interactions is generally ignored.

The majority of natural solid surfaces as well as bacteria are negatively charged (11). In aquatic environments these surface charges are counterbalanced by oppositely charged ions, a part of which is bound to the surface and the remainder distributed in a diffuse layer (16). The thickness of this diffuse layer depends on the ionic strength of the solution and the valencies of the counterions. The electrical interactions between particles (including bacteria) in solution are governed by the extension of the diffuse layer; increasing salt concentration results in a decrease of the electrical interactions between two likewise charged particles.

In the absence of steric contributions due to polymers or polyelectrolytes, the total long range interaction between two likewise charged surfaces is comprised of two additive terms: the electrostatic repulsion and the Van der Waals attraction. Depending on the concentration, the valency and, to a lesser extent the type of the counterions, the repulsion energy can under certain conditions be compensated by the Van der Waals attraction. For more details on this so-called DLVO-theory the interested reader is referred to an article by Rutter and Vincent (16).

There are different possibilities to obtain information about electrostatic interactions. A quantitative method is to determine the electrical potential at each surface. This is experimentally quite difficult. As a good indication of this electrical potential, the determination of the electrokinetic (or zeta) potential is usually sufficient. Under a number of simplifying assumptions, the electrokinetic or zeta potential can be calculated from the electrophoretic mobility. For an exact determination of the zeta potential of bacteria, their conductance needs to be known as well. By ignoring particle conductivity, erroneous results may be obtained, which differ by a factor 0.3 to 0.6 from the real values (4). Einolf and Carstensen (4) found that the conductivity

of bacteria is comparable to that of an 0.01 M NaCl solution. Because of the difficulties to determine the bacterial conductivity accurately, we decided to use in this article the electrophoretic mobility as the measure for the electrostatic state of a bacterium without converting mobilities into zeta potentials. This is a justified procedure for comparison of different bacteria because their conductivities are likely to be very similar.

In this paper we relate the electrophoretic mobility to bacterial adhesion on negatively charged polystyrene. In addition, the influence of the cultivation conditions on the cell surface characteristics have been investigated. Finally the bacterial electrophoretic mobilities were combined with results from hydrophobicity measurements (Chapter 3) in order to obtain quantitative information on the relative contribution of both factors on bacterial adhesion.

## 4.2 MATERIALS AND METHODS

**Growth and preparation of bacterial suspensions.**

Bacteria and preparation of bacterial suspensions are described in Chapter 3. For most experiments bacteria were grown in batch cultures and harvested in the early stationary phase. The growth medium for continuous cultivation was identical with the medium used for batch experiments. The chemostat culture was operated at 25°C. For electrophoretic mobility measurements bacterial cell suspensions were washed twice in an appropriate dilution of phosphate buffered saline (PBS); the last resuspendation was made immediately before measurement to prevent interference of ions leaking from the cells.

**Measurement of electrophoretic mobility.**

Electrophoretic mobility was measured by laser Doppler velocimetry with a ZetaSizer (Malvern Instruments,England). A glass capillary was used as the electrophoresis cell. Bacteria were resuspended in different PBS-concentrations.

## 4.3 RESULTS

For different bacteria a great diversity in electrophoretic mobility and therefore in electrokinetic potential was measured (Table 1). If, as suggested by Einolf and Carstensen (4), conduc-

Table 1 Electrophoretic mobilities for different bacteria, measured in a buffer (PBS) with an ionic strength of $7.5.10^{-3}$ M.

| | Strain | Electrophoretic mobility[a] ($10^{-8}$ m.V$^{-1}$.sec$^{-1}$) |
|---|---|---|
| 1 | *Pseudomonas fluorescens* | -2.36 |
| 2 | *Pseudomonas aeruginosa* | -1.07 |
| 3 | *Pseudomonas putida* | -1.60 |
| 4 | *Pseudomonas* 26-3 | -0.29 |
| 5 | *Pseudomonas* 52 | -2.67 |
| 6 | *Pseudomonas* 80 | -1.74 |
| 7 | *Escherichia coli* NCTC 9002 | -0.42 |
| 8 | *Escherichia coli* K 12 | -1.38 |
| 9 | *Arthrobacter globiformis* | -1.84 |
| 10 | *Arthrobacter simplex* | -1.08 |
| 11 | *Arthrobacter* 177 | -3.24 |
| 12 | *Arthrobacter* 127 | -1.37 |
| 13 | *Micrococcus luteus* | -1.62 |
| 14 | *Acinetobacter* 210A | -1.99 |
| 15 | *Thiobacillus versutus* | -2.97 |
| 16 | *Alcaligenes* 175 | -2.57 |
| 17 | *Rhodopseudomonas palustris* | -2.68 |
| 18 | *Agrobacterium radiobacter* | -1.48 |
| 19 | *Bacillus licheniformis* | -2.40 |
| 20 | *Corynebacter* 125 | -3.07 |
| 21 | *Azotobacter vinelandii* | -2.45 |
| 22 | *Rhizobium leguminosarum* | -2.10 |
| 23 | *Mycobacter phlei* | -3.09 |

[a] average standard deviation ± 0.15 $10^{-8}$ m.V$^{-1}$.sec$^{-1}$

tivity is taken into account in the conversion of mobilities into zeta potentials, the latter range from -10 mV to -90 mV. The electrophoretic mobility was measured as a function of the salt concentration (Fig. 1). Normally the electrophoretic mobility will increase with decreasing salt concentration. However, bacteria conduct part of the current which leads to a reduction of the mobility, particularly when the conductivity of the solution is low. As a result maxima can occur in the mobility-log concentration diagram.

The electrophoretic mobilities of bacteria are combined with the adhesion behavior of bacteria to sulphated polystyrene as reported Chapter 3 (Fig. 2). At the electrolyte strength (0.1 M PBS) used in the adhesion experiments, the electrostatic interactions between bacteria and surface are strongly reduced. A full comparison is not possible because the adhesion measurements have been done in 0.1 M PBS whereas, because of practical limi-

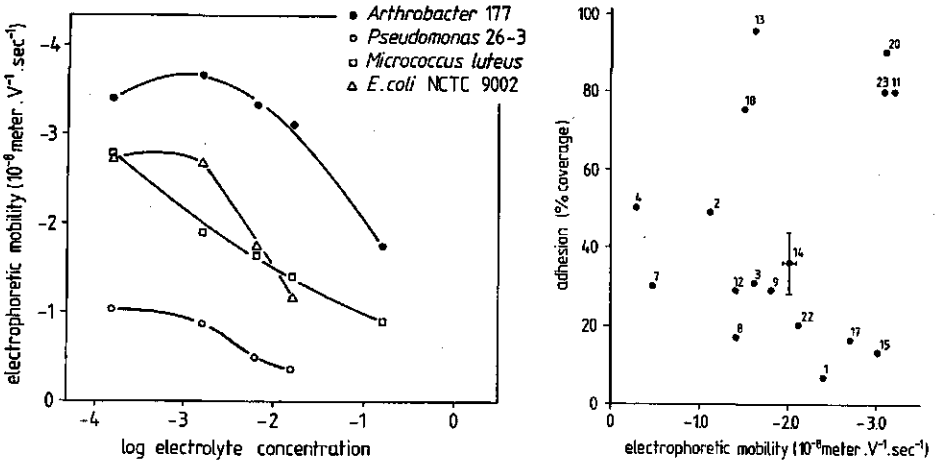


Figure 1 Relation between electrophoretic mobility and electrolyte concentration for four bacterial strains.

Figure 2 Relation between electrophoretic mobility of bacteria (in 0.0075 M PBS) and adhesion to negatively charged polystyrene (in 0.1 M PBS). Numbers refer to the different bacteria in Table1. Bars indicate the average standard deviation.

tations, electrophoresis had to be performed in 0.0075 M PBS. This fact had, however, no significant influence on the results shown in Fig. 2, since the relative range of mobilities remain approximately the same in 0.1 M PBS. A brief comparison between electrophoretic mobility at 0.0075 M PBS and 0.05 M PBS showed no significant differences in the relative range of the bacterial cell electrophoretic mobility (data not shown).

Table 2 Contact angle and electrophoretic mobility of different bacteria grown in batch cultures on various substrates.

| Growth substrate | Bacterial strain *Pseudomonas* strain 26-3 | *Arthrobacter* strain 177 | *Arthrobacter globiformis* | *Escherichia coli*(NCTC 9002) |
|---|---|---|---|---|
| Acetate | 28/-0.4[a)] | 62/-3.2 | 24/-1.8 | -/-[b)] |
| Ethanol | 21/-0.3 | 60/-3.2 | 23/-1.8 | -/- |
| Mannitol | 21/-0.4 | 60/-3.2 | 23/-1.8 | 18/-0.3 |
| Glucose | 21/-0.3 | 64/-3.2 | 23/-1.9 | 19/-0.5 |
| o-Xylene | -/- | 61/-3.1 | -/- | -/- |

a) The first number represents the contact angle of water (12). The value after the slant line gives the measure of the electrophoretic mobility in $10^{-8}m.V^{-1}.sec^{-1}$.

b) -/- no growth of these bacteria on this substrate.

To investigate the influence of growth substrate and growth conditions on the hydrophobicity and electrophoretic mobility of bacteria, two complementary experiments have been performed. In the former, the effect of the various substrates has been measured. Cells were harvested in the early stationary phase. Only small influences of the growth substrate on the surface properties were observed (Table 2). In the latter, the influence of the bacterial growth rate on surface properties was measured

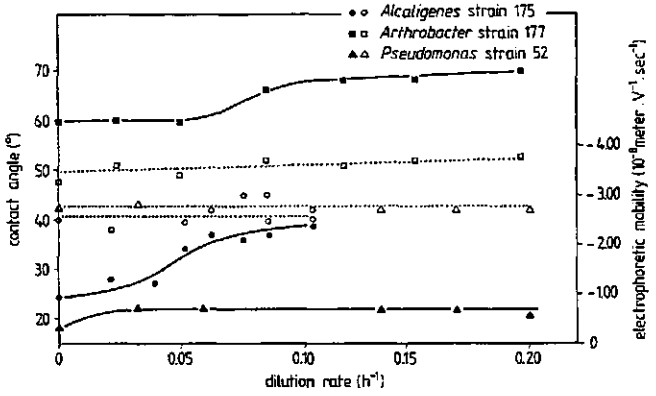


Figure 3 Cell surface hydrophobicity (——), determined with the water contact angle method (12, standard deviation ±1°), and cell electrophoretic mobility (•••••) (standard deviation ±0.15 $10^{-8}$ m/Vs) as a function of dilution rate, in a chemostat.

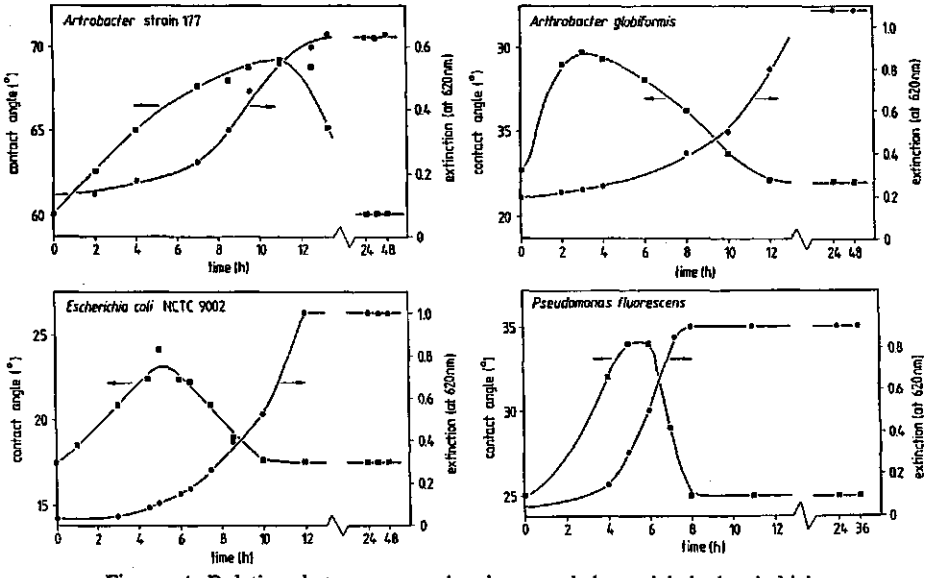


Figure 4 Relation between growth phase and bacterial hydrophobicity.

in a chemostat (Fig. 3). The hydrophobicity increased with increasing dilution rate, while the electrophoretic mobility did not change markedly. Similar results were obtained with batch experiments in which the cell surface of the strains tested increased during the exponential growth phase (Fig. 4).

## 4.4 DISCUSSION

Based on the data given in Fig. 2 it can be concluded that no clear correlation between electrophoretic mobility of bacteria and their adhesion to solid surfaces exists. However, by combining these data with the results from contact angle measurements (Chapter 3) the relative influence of the electrokinetic potential becomes obvious (Fig. 5). This figure was obtained by interpolating the data with a SAS/GRAPH computer program (SAS institute Inc.,Cary N.C., USA). As can be seen from Fig. 5 surface hydrophobicity is the dominant characteristic. At high contact angle for water adhesion complete adhesion is found, irrespective of the mobility. However, at more hydrophilic cell surfaces the electrokinetic potential becomes more influential. This means that bacteria may adhere in the so-called secondary minimum (16). In that case it is impossible to calculate the Gibbs energy of adhesion from a balance of interfacial tensions (1,2), because no phase bounderies are destroyed or formed.

By comparing the data in Table 1 with those on bacterial hydrophobicity reported in Chapter 2, the trend emerged that relatively

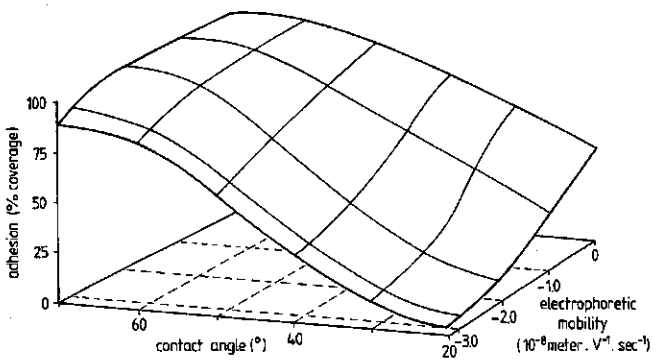


Figure 5 Relation between bacterial adhesion and cell surface characteristics as determined by electrophoretic mobility and contact angle measurements (interpolation of the data in Fig. 2 in this Chapter and in Fig. 5 in Chapter 3).

hydrophobic cells also had high negative electrokinetic potentials. The combination of a high surface potential and a hydrophobic surface seems to be contradictory, but the charged groups only occupy a minor fraction of the total surface area. Assuming all charge is caused by carboxyl-groups on the outer surface at a relatively high surface charge of 100 $mC.m^{-2}$, not more than 8 % of the surface would consist of charged groups. This is probably already an overestimation because the surface potential results only partly from charged groups at the outer surface but also from charged groups situated in deeper layers of the cell wall. The finding that none of the hydrophobic bacteria had a low electrophoretic mobility might be due to the fact that the isolation of a hydrophobic organism with a low electrokinetic potential from a natural sample would be very difficult. These kinds of bacteria would adhere very strongly to surfaces and to each other. The detachment of a single bacterium from other cells or particles is essential in at least one step during the isolation procedure. Therefore, hydrophobic bacteria with low electrokinetic potential could have escaped classical microbiological isolation techniques. Another explanation for the difficulty to find such bacteria could be that hydrophobicity combined with low electrical charge is for an organism ecologically of a considerable disadvantage, since these characteristics prevent spreading and thus colonization of new habitats. Such a competitive handicap could be detrimental for a non-motile microorganism.

The observation that bacteria become more hydrophobic during the exponential growth phase (13) or at high growth rates in a chemostat (Fig. 3) agrees with the experience of many bacteriologists that during continuous cultivation at high dilution rates many bacteria tend to form flocs or stick to surfaces present in the culture vessel. Despite the fact that studying changes in bacterial adhesion behaviour under different conditions may help to explain the role of surfaces in microbial physiology and ecology, only few experiments related to this subject have been published. Fattom and Shilo (5) observed benthic cyanobacteria to become more hydrophobic and adhere to solids under optimal growth conditions. Also Malmqvist (14) found an increase in

cell hydrophobicity during exponential growth. Wrangstadh et al. (17) showed that the production of an extracellular polysaccharide under starvation conditions induced a decrease in cell surface hydrophobicity and thus in the number of adhered cells. A better adhesion of log phase cells was observed by Fletcher (6), Marshall et al.(15) and Zvyagintsev et al.(18). Similar results were reported by Sie (Flotation der Mikroorganismen in einer Laboranlage, Dissertation, Univ. of Hamburg, West Germany, 1985) who measured better adhesion of microorganisms to air bubbles during the exponential growth phase. On the other hand, Kjelleberg and Hermansson (9) reported an increase in hydrophobicity with four out of seven marine isolates upon starvation, and Dawson et al.(3) found a marine *Vibrio* sp. to become more adherent during starvation. Only in this last case adhesion was found to be stimulated by the formation of polymeric fibrils.

From the few observations which have been reported up to now the following hypothesis may put forward. Most terrestial, lacustrine and near shore microorganisms tend to adhere under optimal growth conditions, while some open ocean microorganisms adhere during starvation. Although these findings seem to be contradictory, both behaviors may favor spreading of microorganisms under unfavorable conditions. The detachment of bacteria in soil or sediments during starvation allows an organism to be transported with the pore water, whereas the attachment to particles in an aquatic environment will increase the vertical transport velocity of a microorganism. In both cases detachment or attachment enlarges the chance to reach environments richer in nutrients elsewhere in the soil or in deeper waters and sediments.

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# CHAPTER 5

## BACTERIAL ADHESION: A PHYSICOCHEMICAL APPROACH


### ABSTRACT

The adhesion of bacteria was studied using a physicochemical approach. Adhesion to negatively charged polystyrene was found to be reversible and could quantitatively be described with the DLVO theory for colloidal stability, i.e., in terms of Van der Waals and electrostatic interactions. The influence of the latter was assessed by varying the electrolyte strength. Adhesion increased with increasing electrolyte strength. The adhesion Gibbs energy for a bacterium and a negatively charged polystyrene surface was estimated from adhesion isotherms and was found to be 2-3 kT per cell. This low value corresponds to an adhesion in the secondary minimum of interaction as described by the DLVO theory. The consequences of these findings for the description of natural and technical processes are discussed.


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## 5.1 INTRODUCTION

In many natural and artificial systems metabolically active bacteria are found to be associated with interfaces. Recognition of the importance of bacterial adhesion in various disciplines has led to an exponential increase in research during the past decade (33). The main areas of research in this field are related to the role of bacterial adhesion in the formation of biofilms and biofouling (12,13,25,34) and the surface colonization by pathogenic bacteria (5,7,15,30). Despite the abundance of solid surfaces in soils, publications considering simultaneously adhesion and soil microbiology are scarce. Microbial adhesion in soils has been discussed by Stotzky in a review on soil microbial ecology (38). He stated that interactions between bacteria and soil must be strong. This statement was based on the following observations: (i) during heavy rains only a small number of microbes is transported to underlying soil layers, (ii) in perfusion experiments with soil columns only few microbes are washed out, and (iii) it is necessary to use sonication, surfactants or chelating agents to obtain reasonable microbial counts in soil. Adhesion is not necessarily induced by growth on solid or adsorbed substrates. In fact, in a water-saturated soil column, over 99% of microbes degrading nitrilotriacetate (a non-adsorbing compound) were attached to soil particles (20).

Since bacteria may be considered colloidal particles, their adhesion can be studied as a physicochemical phenomenon (1,5, 23,24), applying colloid chemical principles. Like most natural surfaces, cell surfaces are usually negatively charged and may have varying degrees of hydrophobicity (22,23). Obviously, bacteria are no inert colloidal particles. Their cell surfaces and their characteristics can change with altering environmental conditions in a way that is not usually considered in colloid chemical approaches. For instance an increased substrate flux which influences the growth rate may change the cell surface hydrophobicity (24).

To describe bacterial adhesion as a physicochemical phenomenon, the adhesion Gibbs energy of bacteria ($\Delta_{adh}G^{\sigma}$) can be obtained from a balance of interfacial Gibbs energies:

$$\Delta_{adh}G^{\sigma} = G_{BS}^{\sigma} - G_{BL}^{\sigma} - G_{SL}^{\sigma} \qquad [1]$$

$G^{\sigma}$ is the excess Gibbs energy per unit surface ($J.m^{-2}$), the subscript B stands for bacterium, S for solid and L for liquid. In order to calculate the adhesion Gibbs energy some model considerations have to be made. Up to now mainly two concepts have been used for this calculation.

(I) The concept of short range interactions If adhesion is performed at constant pressure and temperature, and if the molecular composition of the surface does not change, all $G^{\sigma}$'s in eq. [1] can be replaced by the corresponding interfacial tensions ($\gamma$). This concept is restricted to those cases where bacteria and the solid surface are in direct contact and the original phase boundaries are replaced by a new one, namely the bacterium-solid interface. When this new interface is formed, interfacial tensions may be used for a direct estimation of the adhesion Gibbs energy. Many authors have found a good correlation between contact angle measurements (which have been used to estimate the solid/vapor and solid/liquid interfacial tension) and bacterial adhesion (1,5,9,23) and have therefore applied this concept to discuss bacterial adhesion (1,5).

(II) The concept of long range interactions. The DLVO theory for colloidal stability can be used to calculate the interaction Gibbs energy between a particle and a surface as a function of the separation distance (H). The balance of interfacial Gibbs energies in eq. [1] is the basic premise of this theory. The net interaction Gibbs energy is interpreted in terms of Van der Waals interactions (which are usually attractive) and an electric interaction due to the overlap of the electrical double layers at the charged surfaces. The most important parameters determining the van der Waals interaction are the Hamaker constant, which is a material property, the distance (H) between bacterium and substrate, and the geometry of the system. For simple systems there is an approximative relation between the Hamaker constant and the interfacial tension (28, Chapter 2). Since at short range other interactions (e.g. steric repulsion and hydrogen bonding) also play a role, the DLVO theory can only be used if the separation distance (H) between the surfaces is greater than approximately 1 nm.

Bacteria and natural surfaces are usually negatively charged

leading to a repulsive electrostatic interactions between cells and surfaces. This interaction depends on the surface potentials and the thickness of the electrical double layers. The thickness is inversely proportional to the square root of the ionic strength. At high electrolyte concentration or in the presence of polyvalent counterions the electrostatic interaction will be reduced.

If steric factors are absent, as is usually the case for not too low H, the total interaction Gibbs energy $^{\Delta}G(H)$ is obtained by summation of the electrostatic and van der Waals contributions. Three different situations can be distinguished (see also Fig. 1):

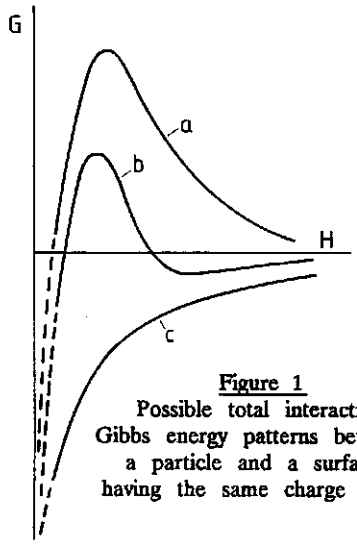


Figure 1
Possible total interaction Gibbs energy patterns between a particle and a surface having the same charge sign.

(a) G(H) displays a high maximum that forms a large barrier against adhesion.
(b)Besides this maximum there exists a secondary minimum in G(H), which is deep enough to result in adhesion at a certain distance from the surface.
(c) G(H) decreases monotoneously with decreasing separation distance; in this case adhesion takes place at a very short distance from the surface.

For a more extensive discussion on the application of the DLVO theory to microbial adhesion the interested reader should refer to Rutter and Vincent (35).

The interaction Gibbs energy between bacteria and surfaces can be assessed from an adhesion isotherm, using e.g. the Langmuir or Volmer theory for adsorption. These theories have the following assumptions in common: (a) adhesion is reversible, (b) adhesion is restricted to a monolayer, (c) the surface is homogeneous, and (d) there is no lateral interaction between adhering cells. The Langmuir theory assumes localized adhesion which means that lateral movement of adhered cells is not accounted for. In the case that the adhered particles are free to move parallel to

the surface, i.e. possess a certain translational Gibbs energy, the Volmer theory should be applied (40). Since adhesion in the secondary minimum of the DLVO theory is not localized the Volmer equation has been used to calculate the interaction Gibbs energy:

$$[\theta/(1-\theta)]\exp[\theta/(1-\theta)] = [X/(1-X)]\exp(\Delta_{adh}G/RT) \quad [2]$$

where $\theta$ is the degree of surface coverage and X the equilibrium volume fraction of bacteria in suspension; R and T have their usual meanings. By omitting the term $\exp[\theta/(1-\theta)]$ the Langmuir equation is obtained. As compared to the Langmuir expression the Volmer equation predicts a higher adhesion at low volume fractions, and lower adhesion at high volume fractions.

This study was undertaken to investigate the applicability of the DLVO theory and the surface Gibbs energy approach for the description of the initial step of microbial adhesion. In the following, both theories are compared with the outcome of adhesion measurements. The conclusions from this comparison are critically evaluated in the light of the adhesion of bacteria in their "natural" environment.

## 5.2 MATERIALS AND METHODS

**Bacterial cultivation**

The cultivation methods were described previously (23). The surface characteristics of the bacteria are summarized in Table 1. *Pseudomonas* strain 62 is identical to *Pseudomonas* strain 26-3 in the previous article(23).

Table 1 Surface properties of the bacteria used in the adhesion experiments[a)]

| Bacterial Strain | Contact Angle (°) | Electrophoretic Mobility ($10^{-8}$ m.V$^{-1}$.s$^{-1}$) |
|---|---|---|
| *Arthrobacter globiformis* | 24 | -1.84 |
| *Arthrobacter* strain 177 | 60 | -3.24 |
| *Escherichia coli* NCTC 9002 | 15 | -0.42 |
| *Micrococcus luteus* | 45 | -1.62 |
| *Pseudomonas* strain 62 | 21 | -0.29 |

a) Details on the measurements are given chapter 3 and 4.

**Labelling of bacteria**

A method described by Puepke (32) was slightly modified and used as adhesion assay. A test tube containing 2 ml of normal growth medium (23) and 450 kBq [L-$^{35}$S] methionine (800 Ci.mmol$^{-1}$, Du Pont) was inoculated with approximately $10^7$ cells and incubated on a rotary shaker at 25°C. After two days the cells were harvested, centrifuged and washed four times at 4°C with 0.1M phosphate-buffered saline (PBS: 0.29 g $KH_2PO_4$, 1.19 g $K_2HPO_4$, 4.93 g NaCl, 1 l demineralized water). The suspension was filtered over an 8 µm membrane filter (Sartorius) to remove cell clumps.

To control the stability of the incorporation of the $^{35}$S-label, suspensions in different PBS-concentrations were incubated for one hour. The suspension was subsequently filtered over an 0.2 µm membrane filter and the radioactivity in the filtrate measured. The filtrate always contained less than 0.1% of the originally incorporated label. Interference by leakage could therefore be excluded. To obtain the specific activity of the cell suspension, the cell concentration was determined with a counting chamber and the radioactivity of 10 µl suspension was measured. Depending on the bacterial strain the specific activity of the cells ranged from 50 to 800 cells/dpm.

**Adhesion assay**

Immediately before the experiment the washed cell suspension was diluted with PBS to obtain the desired bacterial cell concentration. Six 25 µl cell suspension droplets were deposited on the inner surface of a hydrophobic polystyrene petri-dish. After 10 minutes, polystyrene disks prepared as previously described (23), were placed on top of each droplet. The petri dish was covered and incubated at room temperature for different periods of time, varying from five minutes to one hour. At the end of the incubation, the disks were lifted from the droplets and washed carefully in PBS to remove non-attached cells. Care was taken that during the washing procedure no cells were transferred from the polystyrene surface to the water/air interface. The disks were placed in a scintillation vial and the radioactivity was measured with a liquid scintillation counter (LKB) using 4 ml of aqualumen (LUMAC/3M) per vial. Aqualumen solubilizes the bacterial cells as well as the polystyrene; no quenching was observed. The equilibrium concentration of cells in the liquid phase at the end of the experiment was measured by determining the radioactivity in the remaining water droplet (10 µl in 4 ml of aqualumen). The surface coverage of the polystyrene disks was calculated by multiplying the amount of cells per square meter by the cross section

area of the cell, which was quantified by light microscopy.

To measure the influence of the ionic strength of the medium on adhesion, cells were first washed in $10^{-4}$M NaCl solution and then brought into PBS of different concentrations. Adhesion was quantified as described above.

**Detachment assay**

The rate of detachment of adhered cells was tested by bringing disks immediately after the adhesion experiment into contact with sterile PBS. This was done in three ways: (i) by laying the disks on the PBS solution surface (here detachment is a mainly diffusion- controlled process), (ii) by shaking the disks manually in PBS (representing a detachment process by convective transport), and (iii) by washing the disks with a water jet. During this last treatment detachment was almost entirely controlled by surface shear forces. The detachment was followed by measuring the decrease in radioactivity remaining on the disks after various time intervals.

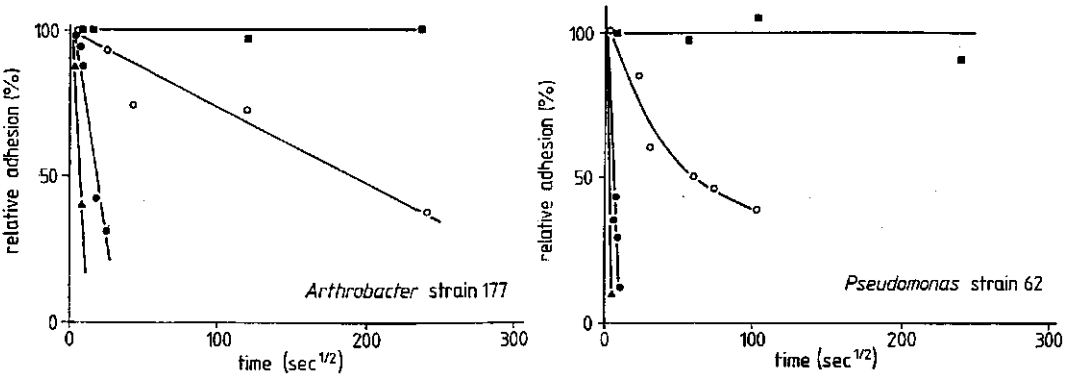


Figure 2 Bacterial adhesion and detachment course. (■) Adhesion, (○) Diffusion controlled detachment, (●) Detachment in a mixed system, (▲) Detachment under strong surface shear. Adhesion is given as the fraction of maximum adhesion. The points represent the mean of three independent experiments. The standard deviation in the adhesion assay was ± 15 %.

## 5.3 RESULTS

In preliminary experiments the adhesion rate and the applicability of the Volmer premises were tested. No differences in adhesion were detected between disks incubated from 5 minutes up to 24 hours (Fig. 2), indicating that adhesion equilibrium was reached within 5 minutes. To ensure having reached equilibrium, in all further experiments an incubation time of 30 min. was

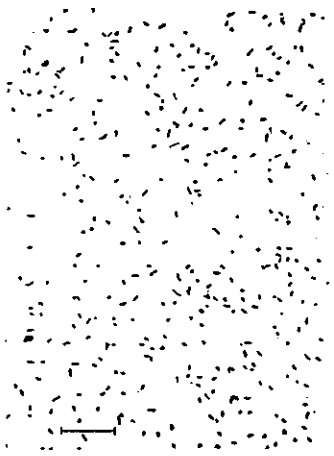

Figure 3 Microscopic view of *Pseudomonas* strain 62 adhering on polystyrene. Bar represents 20 μm.

chosen. Microscopic observations showed that bacteria adhered in a monolayer. Lateral interactions could be neglected because the surface coverage in the experiments was always less than three percent and the bacteria were in all cases randomly distributed (Fig. 3). Reversibility of adhesion was tested by incubating disks with adhering cells on sterile PBS. The number of adhering cells slowly decreased in time (Fig. 2) indicating reversible bacterial adhesion to polystyrene. Reversibility of bacterial adhesion has also been reported by others (5,25,30,32). Above findings indicate that the premises for the Volmer theory, as mentioned in the introduction, are fulfilled. Detachment of cells was also studied under non stationary conditions, i.e. with convective transport of cells and under surface shear. Both treatments increased the detachment rate drastically (Fig. 2).

Table 2 Adhesion Gibbs energy of bacteria, estimated by the Volmer theory.

| Bacterial strain | Surface[a] | $\Delta_{adh}G^{b}$ (kT) | Surface coverage range (%) | Lit. |
|---|---|---|---|---|
| *Arthrobacter globiformis* | P.S. | - 2.5 | 0.001 - 0.7 | |
| *Arthrobacter* strain 177 | P.S. | - 1.9 | 0.005 - 1.5 | |
| *Escherichia coli* NCTC 9002 | P.S. | - 2.1 | 0.002 - 2.5 | |
| *Micrococcus luteus* | P.S. | - 2.5 | 0.020 - 1.2 | |
| *Pseudomonas* strain 62 | P.S. | - 3.1 | 0.020 - 1.7 | |
| *Pseudomonas fluorescens* | P.S.D. | - 6 | 2.0 - 20 | (10) |
| *Escherichia coli* | A.E. | - 6 | 0.1 - 10 | (14) |
| *Streptococcus aureus* | Silt loam | - 4 | 0.01 - 2 | (17) |
| *Streptococcus sanguis* | P.S. | - 4 | 0.1 - 6 | (30) |

[a] abbreviations used: P.S.: polystyrene; P.S.D.: polystyrene petri-dish; A.E.: anion exchange resin; P.C.: polycarbonate.

[b] the Gibbs energy is given for one bacterium.

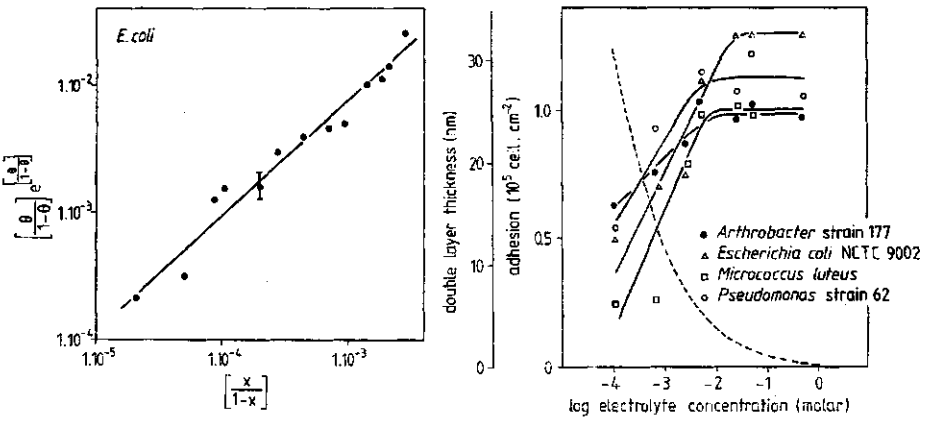


Figure 4 Linearized Volmer isotherm for *E.coli* on negatively charged polystyrene
The points represent the mean of 6 independent measurements. Bar indicates the average standard deviation in the measurement.

Figure 5 Relation between bacterial adhesion and electrolyte concentration. The points represent the mean result of three independent measurements. The standard deviation in the adhesion assay is ± 15 %. (--------): Electrical double layer thickness as a function of NaCl concentration.

An adhesion Gibbs energy of -2.1 kT was calculated from the linearized adhesion isotherm for *Escherichia coli* (plotted in Fig. 4). The adhesion Gibbs energies for the different strains calculated according the Volmer theory, together with results derived from literature, are given in Table 2. The energies calculated from the literature are based on graphical representations of adhesion data and on bacterial dimensions taken from photographs. Since the original data of the published experiments were not available, the calculated adhesion Gibbs energies are merely indicative.

Because of their negatively charged surfaces, bacteria and polystyrene have a negative electrokinetic potential. In Chapter 4 we reported adhesion to decrease with increasing bacterial electrokinetic potential of the bacteria, a strong indication that electrostatic interaction contribute to $\Delta_{adh}G^{\circ}$. Additional evidence stems from the influence of electrolytes. According to the DLVO theory adhesion should decrease with decreasing ionic strength, a prediction that was corroborated (see Fig. 5).

## 5.4 DISCUSSION

Microorganisms can adhere in two ways, viz. either by generic physical-chemical forces (1,5,15), or with the use of specific surface structures of the cell such as pili, fimbriae or other appendages (7,8,16). Adhesion with specific surface structures is usually based on molecular recognition and hence takes place by direct contact only. By approaching a surface from long distance an organism will first be exposed to generic physico-chemical forces (as described by the DLVO theory) before specific interactions become operative. This successive interplay of forces (long range generic followed by short range specific) has been suggested by adhesion experiments with *Agrobacterium* and plant tissue (26,32). In situations where adhesion is not directly expected to be a specific process, for instance in soils and during biofouling, long range interactions are always responsible for the first step in the adhesion of bacteria. Thus, specific adhesion can occur only when the long range interactions (as described by the DLVO theory) are attractive. In the following the applicability of the DLVO or the surface Gibbs energy approach will be discussed on the basis of our experimental data. The findings will also be used to discuss the role of bacterial adhesion in some technical and natural processes.

**Theories to describe bacterial adhesion.**

The value for $\Delta_{adh}G$, as obtained from adhesion isotherms, is compared to the values calculated on the basis of the two concepts mentioned above. The first concept interprets $\Delta_{adh}G^{\sigma}$ in terms of a combination of interfacial tensions [Eq. 1] assuming adhesion to take place at zero separation. Supposing that only 1% of the bacterial surface is in contact with the solid surface, the adhesion Gibbs energy would range from 600 to 6000 kT per cell, assuming $\Delta_{adh}G$ = 0,1 to 1 mJ/m² (1,5,23; 1 kT = $4 \cdot 10^{-21}$J). This value for $\Delta_{adh}G$ is far too high compared to the results of the adhesion experiments given in Table 2. Theoretical and experimental values would be in agreement only in the case that an unlikely small part of the cell surface (< 0.01%) were in direct contact with the solid surface. Such a small contact surface would make macroscopic hydrophobicity data (e.g. as obtained by contact

angle measurements) meaningless for the description of adhesion, since these relatively crude measurements give only a mean characteristic of a large part of the total cell surface. The observation by many authors(1,5,9,23,30,32)thatadhesion is usually a reversible process does indicate that in their experiments the adhesion Gibbs energy is less than 10 kT per particle, a value which roughly represents the border between reversible and irreversible adhesion. From our data and from the results given in the literature it can be concluded that the interpretation of the adhesion Gibbs energy in terms of a balance of surface tensions is inadequate for a quantitative general description of bacterial adhesion.

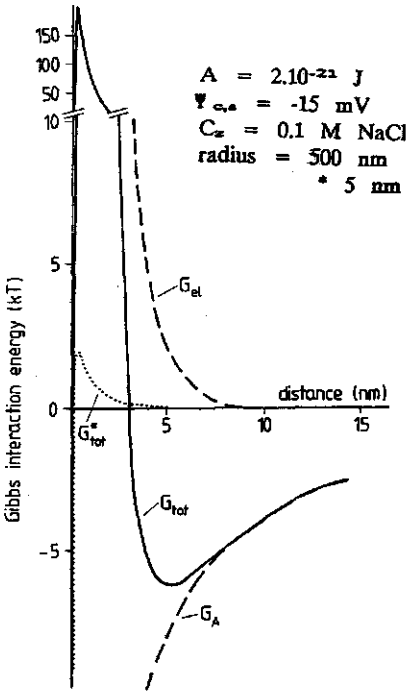


Figure 6 Theoretically calculated DLVO interaction curve for a spherical particle and a flat surface with the same charge.

The DLVO theory formulates $\Delta_{adh}G^{\sigma}$ as a function of separation, taking into account the Van der Waals ($G_A$) and electrostatic ($G_{el}$) energies. Using the equations given by Rutter and Vincent (35) the total interaction Gibbs energy between spherical bacteria and a flat polystyrene surface can be computed (Fig. 6). As a precise quantitative evaluation may be doubtful with respect to bacterial adhesion, only a semi-quantitative description is obtained. For a range of Hamaker constants (0.4 to 6 $10^{-21}$J) and electrokinetic potentials (-3 to -50 mV) the total interaction curve shows a secondary minimum at 4 to 6 nm separation distance and a maximum exceeding 50 kT at 1 nm from the surface. This maximum generally prevents (irreversible) adhesion of the whole cell in the primary minimum. The Gibbs energy at the secondary minimum (-1 to -20 kT, depending mainly on the van der Waals interaction) calculated with the DLVO theory, is of the same order of magnitude as the experimentally

obtained adhesion Gibbs energy (Table 2). The finding that bacteria adhere at a certain distance between the surfaces (secondary minimum) where the cells are free to move parallel to the surface justifies the application of the Volmer theory to calculate the adhesion Gibbs energy. Recently the existence of a certain distance between adhering bacteria and surface has been shown by interference reflection microscopy (10a).

**Relation between cell surface parameters and adhesion.**

A number of authors have interpreted the adhesion Gibbs energy in terms of the interfacial tensions $\gamma_{SB}, \gamma_{BL}, \gamma_{SL}$ of the three phase boundaries involved. This is obviously at variance with the evidence for adhesion in the secondary minimum, as presented above. The observation that the mentioned theories are internally consistent may be due to the fact that $\gamma_{SB}, \gamma_{BL}$ and $\gamma_{SL}$, which are experimentally inaccessible quantities, are obtained by invoking some model considerations in interpreting contact angle data; these data are to a large extent determined by Van der Waals interactions (28), as is also the case for secondary minimum adhesion (Fig. 6). The internal consistency (i.e. good correlation between contact angles and adhesion) is therefore not a justification for the substitution of $G^{\sigma}$ by $\gamma$.

Although to a lesser extent, electrostatic interactions do also play a role in bacterial adhesion (24). This can for instance be deduced from the increased adhesion when the electrolyte strength increases (12,25, Fig. 5). The effect of the electrolyte concentration can be explained by the theory for the overlap of diffuse double layer theory (35). The thickness of the diffuse layer of counter charge surrounding a charged particle is a function of the ionic strength. With increasing ionic strength this thickness decreases (Fig. 5), thereby changing the position and depth of the secondary minimum. In fact, an increased or even irreversible adhesion in the presence of multi-valent counterions is often observed (2,10a,13,25,29,34,39). In colloid chemistry the pronounced effect of the valency of the counterions on electrostatic interactions can, e.g., be inferred from the concentrations needed to destabilize a negatively charged AgI sol: 140 mM for $NaNO_3$, 2,4 mM for $Ca(NO_3)_2$ and 0,067 mM for $Al(NO_3)_3$ (19). Thus, in adhesion experiments the concentrations

of di- and tri- valent cations should be carefully controlled.

**Reversible versus irreversible adhesion.**

In this as in many other bacterial adhesion studies a relatively low adhesion Gibbs energy (Table 2) and, consequently, reversible adhesion was found. As discussed above, this observation can be interpreted as adhesion in the secondary minimum of the DLVO interaction curve. It is suggested that motile bacteria may be able to overcome the energy barrier between secondary and primary minimum due to their kinetic energy and consequently may adhere irreversibly. In excess to the energy of normal thermal motion (1.5 kT) motile bacteria possess a kinetic energy that usually does not exceed 1 to 1.5 kT (bacterial velocity 100 $\mu m.sec^{-1}$, (36)). This is not sufficient to pass energy barriers as high as 50 kT. Nevertheless, in some cases irreversible adhesion is observed. This might occur when bacteria adhere in the primary minimum as described by the DLVO theory, or if the energy in the secondary minimum is sufficiently negative. Primary minimum adhesion (the interaction energy of which cannot be calculated by the DLVO theory) is possible only if the maximum in the $\Delta G(H)$ curve is absent or does not exceed a few kT units. This is the case when:

(i) the surface is positively charged (21,27,34). Since bacteria are negatively charged, surface and bacteria will electrostatically attract each other. However, in nature positively charged surfaces are very rare. Biopolymers or small anions like phosphates or silicates will immediately bind to them and consequently render the effective charge negative (22).

(ii) both the bacterium and the surface are hydrophobic (i.e. strong van der Waals interaction) and low charged. In Chapter 4 a 100 % surface coverage of negatively charged polystyrene by hydrophobic low charged cells was predicted. Busscher et al. (6) showed that irreversible adhesion only occured when surface and bacterium were both hydrophobic.

(iii) high electrolyte strength or di- or tri- valent cations are present (2,29,34).

(iv) bacteria have special surface appendages (e.g. pili or fibrils) that can cover the distance between cell and surface. Due to the smaller radius of curvature of the end of these appendages, repulsion between these structures and the solid surface will be diminished (Fig. 6). For instance, Agrobacterium tumefaciens has been shown to produce cellulose fibrils after adhesion to carrot cells. These fibrils anchor the cells to the surface (26); similar observations have been made with *Rhizobium leguminosarum* (37).

(v) polymers are produced by adhering bacteria. These polymers can relative easily bridge the gap between the cell and the surface (25).

**Implications of bacterial adhesion for several technical and natural processes.**

Because the initial adhesion of bacteria is usually found to be reversible (this study,1,5,9,23,25,30,32) and thus relatively weak, surface shear forces may have a great influence on the initial phase of bacterial adhesion (13, Fig. 2). Powell (31) found for the surface shear that the force parallel, rather than the force perpendicular to the surface governs detachment. The parallel force exerted on the cell is proportional to the square radius of the particle. Since the attractive force is in first approximation linearly proportional to the particle radius, shear forces are more effective in detaching large particles, such as bacteria, than smaller ones. Shear forces acting on reversibly adhering cells will decrease the mean residence time of cells on the surface, however, without changing the average number of adhered cells. The shorter residence time of the individual cell reduces the probability that microorganisms become irreversibly attached (e.g. by simply passing the energy barrier or by bridging the distance between cell and surface with a polymer) in the second stage. When shear forces are applied under conditions where cells can be washed out, desorption of cells is increased causing an increase in the cellular wash-out rate. Thus, initial processes in biofilm formation often depend more on the roughness (which can minimize the effect of shear

forces) than on hydrophobicity or the charge of the solid surface (3,4).

The strong influence of hydrodynamic forces on adhered bacteria explains why in the adhesion assay care should be taken to keep surface shear to a minimum during the washing procedure. The considerable standard deviation usually found for adhesion experiments (15% to 25%, 15,18,23,30 and this study) is probably partly caused by difficulties in exactly standardizing the washing procedure.

Because of the very large area of interface area in soils (water/ soil, water/air, water/plant roots) bacterial adhesion is of special importance to soil microbial ecology. If in water-saturated soil all bacteria adhere reversibly, then, as calculated from the Volmer theory, more than 98% of the population will be found at the solid/ liquid interface (values: pore fraction 40%, specific surface area 1 $m^2.gr^{-1}$, soil density 2.5 $kg.m^{-3}$, $\Delta_{adh}G$ = -3 kT per cell). Thus, it can be assumed that in soil most cells are attached due to the large surface to volume ratio of the inorganic phase rather than due to a strong (specific) interaction between bacteria and soil particles. Therefore, the observation that 99% of a non-sorbing substrate is degraded by attached bacteria (20) is not surprising. Moreover, from the finding that during heavy rains only a small fraction of bacteria is transported to deeper soil layers (38) it may not be concluded that there is a strong (irreversible) bacterium-solid interaction.

The selectivity of the interaction between bacteria and plants has led to the general believe that adhesion between the plant root and the bacterium itself is a specific process caused by a biochemical interaction. The positive influence of $Ca^{2+}$ on the adhesion of *Rhizobium leguminosarum* was e.g. explained by postulating the presence of a $Ca^{2+}$-dependent adhesin (37); however it is also possible that $Ca^{2+}$ deficiency results in a changed cell surface less liable to physico chemical adhesion. More general investigations of bacterial adhesion to plants revealed that the first adhesion step is presumably also a physical process. Puepke (32) showed that adhesion of a *Rhizobium* strain to the roots of a potential host-plant is not related to

the capability of the given strain to infect this root. Moreover, no saturation occured even at $10^9$ cells.$ml^{-1}$. Initial adhesion of *Agrobacterium* to potato tissue (18,32) and *Azospirillum* to corn roots (11), was also found to be a physical rather than a biochemical process. Although a detailed discussion on this subject is beyond the scope of this article, it seems reasonable to suggest that bacteria will adhere to plant roots in the same way as they do to other surfaces. However, infection occurs only then when the adhered bacterium recognizes specific host receptors on the root surface. That is: when the first, generic adhesion step is followed by a second, specific step.

In conclusion, we can say that the initial step in bacterial adhesion is often a reversible process, which in terms of the DLVO theory can be described as secondary minimum adhesion. The DLVO theory might be of use for the understanding and interpretation of several microbial processes like transport of bacteria in the soil, initial biofilm formation, formation of anaerobic sludge granules, and plant- bacterium interactions.

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# CHAPTER 6

## USE OF THE DLVO THEORY IN THE INTERPRETATION OF BACTERIAL ADHESION


### ABSTRACT

In the previous chapters we have shown that the DLVO theory can be used to interprete bacterial adhesion to a model-surface of sulphated polystyrene. In this chapter we have tested in how far the DLVO-theory can be generally applicated to bacterial adhesion. To this end we studied adhesion to (i) glass, as a model for hydrophilic and natural surfaces of silicates and oxides, (ii) polystyrene covered with proteins, as a model for a surface coated with an organic layer, and (iii) Rhine river sediment, as a representation of a natural system. In all these cases adhesion could be interpreted in terms of the hydrophobicity and electrical properties of the surfaces, in accordance with the DLVO theory.


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## 6.1 INTRODUCTION

Bacterial adhesion is an important aspect of biofouling, biotechnological processes and soil microbiology. Recent studies have shown that the initial adhesion process can be described by physical-chemical theories (1,2,7,12,15,20), in particular by a surface Gibbs energy (1,2) or a DLVO type (12,13,22) approach. Previously it was found that bacteria differ widely in (i) hydrophobicity which is related to the surface Gibbs energy and van der Waals interaction in an aqueous environment (10), and (ii) electrophoretic mobility which is a measure for the electrostatic interaction, (11). Hydrophobicity is measured through the contact angle of water on a layer of bacterial cells. This contact angle is determined by the difference in internal molecular interactions in the solid and the liquid. If the solid surface Gibbs energy is smaller than the surface Gibbs energy of water (72 $mJ/m^2$) then it is possible to say that: the greater the difference in molecular interactions between solid and water the smaller the solid surface Gibbs energy and the greater the Van der Waals interaction between two solid surfaces in water.

Adhesion to sulphated polystyrene (hydrophobic, negatively charged) appeared to be influenced both by the surface hydrophobicity and electrophoretic mobility (Chapter 3 and 4). The hydrophobicity had the most pronounced effect; the influence of the electrokinetic potential increases with decreasing bacterial hydrophobicity. The results of these and other experiments could best be described by the DLVO theory (Chapter 5). If only van der Waals and electrostatic interactions contribute to adhesion, this theory can predict whether the interaction between cell and surface is weak (reversible) or strong (irreversible). Instead of accounting for both Van der Waals and electrostatic interactions, adhesion has often been discussed in terms of either one of these interactions. This makes it difficult to compare the relative influences of the two types of interaction from literature.

In many natural systems dissolved organic matter is present besides bacteria and solids. This organic material may adsorb onto the bacterial and/or solid surfaces and thereby influence bacterial adhesion. This influence has clearly been shown by

Dexter (5) in a comparison between in situ and in vitro adhesion studies. Polymers may influence bacterial adhesion in four distinguishable manners:

i The presence of the adsorbed layer must be accounted for in the Van der Waals interaction ($G_A$).

ii If the charge density of the adsorbed layer differs from that of the bare surface, the electrostatic interaction ($G_{el}$) may change.

iii When polymers adsorb and coat both bacteria and solid surface completely, an extra repulsive interaction ($G_S$) may be introduced in the DLVO theory due to steric hindrance. This is schematically shown in Figure 1.

iv If one or both surfaces are partly covered with polymer, then, on approach of the two surfaces, one and the same polymer molecule may attach to both surfaces, thereby forming a "bridge" between the two surfaces. This involves a Gibbs energy effect as indicated in Figure 2.

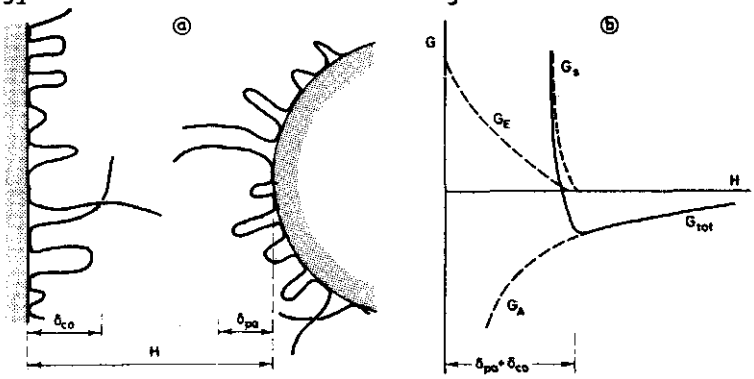


Figure 1 Interaction between like-charged polymer coated-surfaces

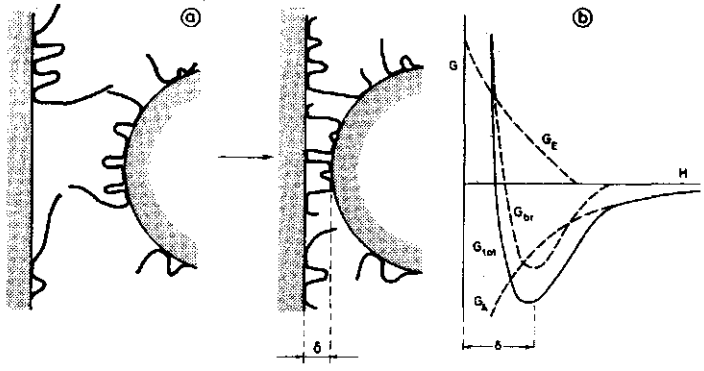


Figure 2 Polymer bridging between like-charged surfaces.

For features (iii) and (iv) loosely structured layers are required, and the chains must protrude into the solution over a distance ($\delta$) exceeding the thickness of the electrical double layer. In this case the adsorbed layers on the approaching surfaces interfere before the electrical double layers overlap. If this is not the case the effect of an adsorbed layer can be treated as specified under i and ii.

Only a relatively small amount of literature, published in journals of various disciplines, is available on the effect of adsorbed molecules on bacterial adhesion. The type of compounds used are often surface active agents (5,8,18). These compounds reduce adhesion, especially in the case of hydrophobic surfaces. Larsson et al.(9) deposited fatty acids on a hydrophobic surface by the Langmuir-Blodgett technique. If the hydrocarbon tails are oriented towards the solution adhesion is unaffected, whereas adhesion is completely inhibited when the carboxyl groups are oriented towards the solution. This indicates that not only the type of adsorbed compound but also its orientation at the interface has a great influence on adhesion.

The influence of proteins on adhesion has been regularly studied (6,14,19). Meadows (14) reported that the adhesion to glass is stimulated by casein and gelatine and decreased by protamine and BSA. Fletcher (6) found that the adhesion of a marine *Pseudomonas* to polystyrene decreased due to the presence of BSA, gelatine, fibrinogen, protamine and pepsine. When free proteins were present during the attachment the strongest influence on adhesion was observed; presumably due to protein adsorption on both, the bacterial and polystyrene surface. Pretreatment of the polystyrene surface with proteins also led to a reduction of the adhesion (except with protamine), whereas the pretreatment of bacteria resulted in a decreased adhesion for BSA-treated cells only. Probably, the adsorption of proteins has a greater influence on the hydrophobicity of the polystyrene surface than on the surface of the bacteria. Nevertheless, Miörner et al. (16) have clearly indicated that proteins (HSA, fibrinogen and immunoglobulin G) influence the surface properties of bacteria. The fact that proteins not only influence the hydrophobic but also the electrostatic interaction may be inferred

from the observation that the basic proteins protamine and histone (which are positively charged at pH 7) have no influence on adhesion when adsorbed on polystyrene (6); presumably a decreased Van der Waals attraction is balanced by a decreased electrostatic repulsion.

In this study the relative importance of hydrophobicity and electrokinetic potential for bacterial adhesion to various surfaces will be investigated and the general applicability of the DLVO theory for the interpretation of bacterial adhesion discussed.

## 6.2 MATERIALS AND METHODS

**Bacterial cultivation**

Bacterial strains and cultivation methods were described in Chapter 3. *Arthrobacter* strain 4-2 is a spontaneous mutant of *Arthrobacter* strain 177 deficient in xylene degradation.

**Surfaces**

Polystyrene. The polystyrene disks were prepared from a polystyrene latex as described in Chapter 3.

Glass. Glass cover slips (Chance propper LTD, Warley UK) were cleaned for 24 hours in chromic-sulfuric acid, and thereafter rinsed with demi-water, 0.1 N NaOH and again demi water. The glass was dried and stored dust-free.

Rhine-sediment. The sandy sediment was collected from the river Rhine near Wageningen, The Netherlands. The sand was sieved to remove stones and particles larger then 2 mm. The sediment contained 0.05 % organic carbon and almost no clay minerals.

Coating with proteins The polystyrene was coated with proteins by incubating disks in a 1 g/dm$^3$ solution of Bacitracin (Sigma 0125), Gramicidin (Sigma 5002), BSA (Sigma 6003), Gelatine (Merck 4078), RNA-se (Sigma 5000), Lysozyme (Merck 5282) and K99-fimbriae (21). The concentration of protein was sufficiently high to obtain complete surface coverage (17).

**Determination of surface characteristics**

Hydrophobicity. Bacterial hydrophobicity was determined as the contact angle of water on a layer of cells (Chapter 3). The influence of proteins on the hydrophobicity of the polystyrene surface was also determined by measuring the contact angle of water on the protein-coated polystyrene.

Electrophoretic mobility. The determination of the bacterial electrophoretic mobility has been described in Chapter 4. To determine the influence of proteins on the electrokinetic potential of the polystyrene, the latex, from which the polystyrene disks were prepared, was coated with the proteins. After protein adsorption the latex was washed twice and the electrophoretic mobility of the coated particles determined.

**Adhesion Assay**

Polystyrene. Bacterial adhesion to (protein-coated) polystyrene was determined with $^{35}S$-labelled cells as described in Chapter 5.

Glass. Bacterial adhesion to glass was determined microscopically. A drop of suspension (1-3.$10^9$ cells/ml) was deposited on a cleaned object glass and covered with a clean cover slip. The amount of adhered cells was determined by focussing the microscope on the cover slip/water interface and counting the amount of cells per view area at 20 different places on the cover slip.

Rhine sediment. Hungate tubes (16 ml screw capped reagens tubes) without and with 3 gram sediment were dry sterilized. A bacterial culture at the end of the exponential phase was washed in 0.1 M PBS (10) and resuspended in 0.1 M PBS at a concentration of approx. 5.$10^8$ cells/ml. From this suspension a serial dilution was made up to approx. $10^2$ cells/ml. From each dilution 5 ml was incubated in hungate tubes with or without sediment for 2 hours on an end over end mixer at 4°C. Hereafter the amount of free cells was determined by the plate dilution technique. Finally the average ratio of free cells to attached cells was determined.

## 6.3 RESULTS

The relation between cell surface characteristics and bacterial adhesion to glass is shown in Figure 3. This figure is obtained by interpolating the datapoints for the adhesion of 17 different strains as previously described (10). It should be noted that since we did not have low charged, hydrophobic, bacteria extrapolation to that domain in Figure 3 is less accurate.

As a simulation of natural occuring organic coatings we have coated polystyrene with different proteines. Table 1 summarizes the changes of the physical chemical surface properties of the polystyrene surface resulting from protein adsorption together with the effects on the adhesion of nine different bacterial

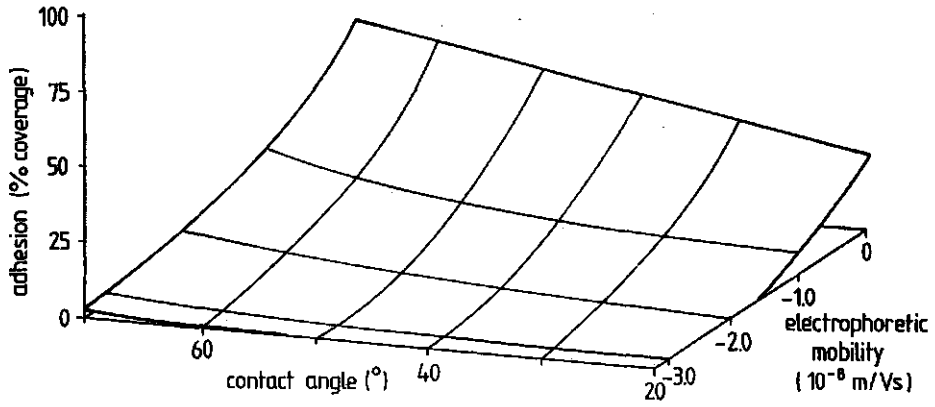


Figure 3 Relation between bacterial adhesion to glass and cell surface characteristics as determined by electrophoretic mobility and contact angle measurement.

strains. The adhesion is expressed as the fraction adhering cells compared to adhesion to the bare polystyrene surface.

Finally, the adhesion of bacteria to river sediment of four hydrophilic and two hydrophobic strains has been studied. Adhesion was determined at different cell concentrations, and an example of such an adhesion isotherm is given in Figure 4. For all other strains adhesion also varied linearly with the equilibrium cell concentration. The adhesion affinities (given as the slope of the adhesion isotherm) for the different strains are collected in Table 2. We have also indicated the amount of cells which will be unbounded (i.e. in the pore water) in the original sediment.

## 6.4 DISCUSSION

From our experiments on bacterial adhesion to sulphated polystyrene we have concluded that the adhesion process can well be described by the DLVO theory, i.e. as the sum-effect of Van der Waals and electrostatic interactions (Chapter 4). In the present study we have observed the influence of these two contributions to the adhesion of bacteria to glass, which is more hydrophilic than polystyrene. Due to the chromic acid cleaning the glass has obtained a high surface charge density (mainly due to oxide groups), and has become very hydrophilic (water contact angle = 0°). It is therefore expected that adhesion to this clean glass is predominantly determined by electrostatic interaction. This is confirmed by the pattern displayed

in Figure 3 where a strong influence of bacterial electrophoretic mobility and a weak effect of contact angle on adhesion is shown. The strong influence of electrostatic interactions on bacterial adhesion to glass implies that glass will preferentially be colonized by low-charged cells. Actually we found that a *Pseudomonas fluorescens* isolated by Caldwell et al.(3) on its ability to rapidly colonize new glass surfaces has a low electrophoretic mobility (- 0.4 $10^{-8}$ m/Vs in 0.0075 M PBS).

Hydrophobic, highly charged bacteria show the greatest difference in adsorption behaviour between glass and polystyrene surfaces. As compared to hydrophilic, uncharged bacteria they adhere in larger amounts to polystyrene but to a lesser extent to glass. This indicates that a bacterium which is the best adhering to one surface is not necessarily the best adhering to all other surface types.

As mentioned in the introduction, a polymeric coating may affect adhesion in two ways: (i) an influence through changes in $G_{AB}$ and $G_{LW}$ (DLVO theory), (ii) an effect resulting from polymer bridging or steric repulsion. In this study we have used proteins as a model for such a coating. The proteins are probably adhering in a compact layer, which implies that their influence may in first approximation be discussed as changes in Van der Waals and electrostatic interactions. The hydrophobicity of the polystyrene surface decreases when proteins are adsorbed (Table 1), which results in a reduced Van der Waals interaction. The electrophoretic mobility of the original polystyrene latex particles also decreases due to the presence of proteins. Although the surface properties of the latex particles are not exactly the same as those of the polystyrene disks, the change in electrophoretic mobility of the protein-coated latex particles probably gives an indication of the effect of proteins on the charge of the disks.

The results for the adhesion of cells to protein-coated polystyrene seem at first glance confusing. However, the general trend in the observations can be explained by the DLVO theory.

Table 1 Influence of proteins on physico chemical surface characteristics (water contact angle θ, and electrophoretic mobility u) of polystyrene, and bacterial adhesion.

| | | Organism strain | *E. Coli* | *Pseudomonas* | | | | | *Arthrobacter* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 52 | 62 | 25 | 8 | 102 | 127 | 4-2 | 177 |
| | | θ (°) | 16 | 19 | 20 | 25 | 25 | 60 | 38 | 40 | 60 |
| Protein | θ (°) | u ($10^8$m/Vs) | -0.4 | -2.7 | -0.3 | -0.2 | -1.8 | -1.9 | -1.4 | -3.6 | -3.2 |
| Control[a] | 70 | -7.8 | 100[b] | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Gramicidin | 70 | -6.6 | 93 =[c] | 90 = | 107 = | 95 < | 102 = | 109 = | 72 < | 132 > | 116 = |
| Bacitracin | 40 | -6.2 | 70 = | 60 < < | 77 < | 55 < < | 84 < | 54 < | 77 < | 40 < < | 87 = |
| Lysozyme | 30 | -0.8 | 98 = | 76 < | 68 < | 62 < < | 107 > | 80 < | 97 = | 552 > > | 159 > |
| K-99 fimbriae | 29 | -... | 80 = | n.d. | 72 < | 53 < < | 76 < | 36 < < | 64 < | n.d. | 76 = |
| RNAse | 24 | -4.1 | 46 < | 68 < | 78 < | 60 < < | 95 = | 74 < | 105 = | 64 < | 111 = |
| BSA | 21 | -6.3 | 23 < < | 75 < | 52 < < | 58 < < | 56 < < | 40 < < | 59 < | 43 < < | 51 < |
| Gelatine | 13 | -0.8 | 67 = | 76 < | 66 < | 78 < | 104 = | 60 < | 78 < | 392 > | 55 < |

[a] Control is untreated sulphated polystyrene

[b] Data are given as index numbers (control = 100)

[c] Different symbols indicate significant differences from the individual controls (LSD test $p < 0.05$)

= : equal adhesion as to control, < less adhesion then to control, < < much less adhesion than to control

> : more adhesion then to control, > > : much more adhesion then to control.

The influence of Van der Waals interactions can be inferred by comparing adhesion to bacitracin coated, BSA coated and bare polystyrene: adhesion decreases with decreasing contact angle. On the other hand, the influence of electrostatic interactions can be traced by comparing adhesion to BSA, and gelatine or bacitracin and lysozyme coated polystyrene: adhesion generally increases with decreasing electrostatic repulsion. A protein like BSA strongly decreases the Van der Waals interaction but leaves the electrostatic interaction almost unaffected; this induces a strong decrease in adhesion. Lysozyme decreases both the Van der Waals and electrostatic interaction which results in an increased adhesion of hydrophobic, high charged cells. The effect of protein coating on the adhesion of *Pseudomonas fluorescens* is great because this bacterium is almost non-charged. Thus, the decrease in Van der Waals interaction is not compensated for by a decreased electrostatic repulsion. On the other hand *Arthrobacter* strain 4-2 is relative hydrophobic and highly charged. This results in a decreased adhesion if only the Van der Waals interaction is decreased. If, however, electrostatic interaction is also strongly reduced adhesion is stimulated.

As an example of a naturally occuring system we have tested the adhesion of 6 different bacteria to Rhine river sediment. This sediment consists mainly of silicates, and is hydrophilic in nature. In contrast to the glass surface, the electrokinetic potential of the sediment particles is less, because they are

Table 2 Relation between contact angle (θ), electrophoretic mobility (U), and bacterial adhesion to Rhine sediment.

| Organisms | θ (°) | U ($10^8$m/Vs) | K[a] (ml/gr) | Cells free in soil solution (%)[b] |
|---|---|---|---|---|
| *Escherichia coli* | 15 | -0.4 | 0.9 | 23 |
| *Pseudomonas* strain 62 | 20 | -0.3 | 20 | 1 |
| *Alcaligenes* strain 175 | 23 | -2.7 | 0 | 100 |
| *Pseudomonas* strain 84 | 25 | -0.2 | 3.4 | 6 |
| *Pseudomonas* strain 102 | 60 | -1.9 | 10 | 2 |
| *Arthrobacter* strain 177 | 60 | -3.2 | 3.5 | 6 |

[a] K = slope of adhesion isotherm as given in Figure 4
[b] Calculated with the following assumptions: pore water fraction 40 %, sediment density 2.5 mg/ml.

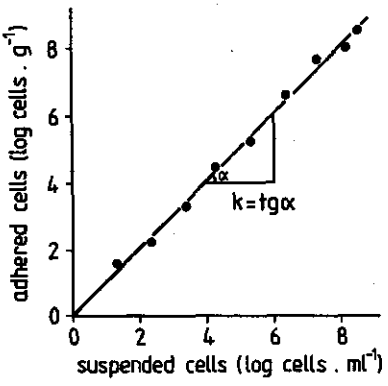


Figure 4 Adhesion isotherm for *E. Coli* on river sediment.

not treated by chromic acid. This implies that the adhesion pattern of bacteria to the sediment will be in between that to glass and to sulphated polystyrene. Like with glass the hydrophilic, highly charged *Alcaligenes* strain 175 is almost completely repelled from the surface. In contrast, other hydrophilic but low charged strains adhere well to the sediment. Comparison of the adhesion of both hydrophobic strains also reveals the influence of electrostatic interactions. Adhesion to the sediment differs from adhesion to glass in that hydrophobic, charged cells adhere to the same extent as hydrophilic, uncharged cells. This indicates that for adhesion to the sediment, the hydrophobic interaction is of more significance than for adhesion to glass, but less than for adhesion to sulphated polystyrene. It should be noted that the transport of cells in a sediment or soil cannot be described by only measuring adhesion isotherms (as e.g. with organic substances), because the transport of cells will also be influenced by a filtration effect.

In conclusion, with well-defined clean surfaces (polystyrene and glass) but also under more natural conditions (river Rhine sediment, protein-coated surfaces) the early stages of bacterial adhesion can be described and understood in terms of the DLVO theory. Applying physical chemistry to the study of microbial adhesion can give, at least at a conceptual qualitative level, a good insight in the occuring phenomena.

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# CHAPTER 7

## THE INFLUENCE OF INTERFACES ON MICROBIAL ACTIVITY


### ABSTRACT

Bacterial adhesion is ubiquitous in natural and artificial systems. Addition of a solid phase to a bacterial culture has been shown to trigger changes in the activity of some cultures. Mostly observed changes can be explained in terms of substrate availability. A decrease in activity of adhered cells may be due to diffusion limitation from nutrients to the surface. Contraryly, an increased activity of adhered cells may be the result of enhanced desorption of adsorbed nutrients. Substrate adsorption leads to a decreased concentration in solution, and as a consequence to a decreased microbial activity. Adsorption of toxic substrates, compounds or intermediates allow an increase in bacterial activity. When the substrates are strongly adsorbed their bioconversion becomes desorption limited, or will not take place at all. The presence of a solid phase (especially clay minerals) may have several other indirect physical, chemical and/or biological effects such as pH buffering, increased survival of the microbes, increased microbial productivity, or increased DNA-transformation. Based on the data reported in the literature and thermodynamic and kinetic relationships it must be concluded that there exist neither theoretical nor experimental evidence for a direct influence of the presence of an interface on the bacterial metabolism.


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## 7.1 INTRODUCTION

In contrast to natural environments where a wide variety of surfaces is available for attachment and colonization by micro-organisms, bacteria are in the laboratory generally cultivated in liquid media in suspension. In nature, however, solid surfaces appear to be the major site of microbial activity. By staining actively respiring bacteria with tertrazolium, Harvey et al. (36) showed that in a marsh estuary, almost all the detectable respiring bacteria were associated with particles. Glucose mineralization in an estuary is also predominantly carried out by adhered bacteria (32). In several ponds and marshes the contribution of particle bound bacteria to total heterotrophic activity has been found to be at least four times as high as could be expected on basis of the fraction of attached cells (56). Also in soils, degradation of a non-adsorbing compound was found to be carried out essentially by attached organisms only (57).

Bacterial adhesion is not only of importance in microbial ecology but also in biotechnology, biofouling, caries formation or (aerobic and anaerobic) waste water treatment. In 1913 Söhngen (88) already reported that a solid phase influences a diversity of bacterial processes like: nitrogen fixation, alcohol oxidation, nitrification and denitrification. In later years more detailed studies on the relation between bacteria and solid surfaces have been reported. Zobell (101), Heukelekian and Heller (46) showed an increased bacterial activity in the presence of glass surfaces, especially at low nutrient concentrations. Bacterial activity in soils in relation to the presence of clay minerals, has been thoroughly studied by Stotzky (89,90,91,92). The influence of anion exchange resins (38,39,40,41) or plastics (8,9,22,24,25,26) on adhesion and activity of bacteria has been studied for a variety of cases in detail. A general consensus seems to exist that surfaces influence bacterial growth (7). However, no consistent pattern of changes in activity is discernible, nor is there a general explanation for the influence of surfaces on bacterial activity (7). This inconsistency is probably due to the great variation in experimental design with respect to the solid phase,

Table 1 Summary of the literature on the influence of solid surfaces on microbial behaviour.

| Observation | Explanation | Reference |
|---|---|---|
| Increased growth | Increased substrate concentration at the interface | 46,101 |
| | More efficient use of proton motive force | 19 |
| | Detoxification of substrate. | 18,72,92 |
| | or inhibitors | 22,37,92 |
| | pH buffering by ion exchange | 14 |
| | no explanation | 26,39,40, 42,52,53,88 |
| Decreased growth | Less cell surface is available for substrate uptake | 55,51 |
| | Higher maintenance coefficient | 55 |
| | Substrate transport limitation. | 13 |
| | no explanation | 5,80 |
| Increased assimilation and decreased respiration | no explanation | 9 |
| Decreased assimilation | no explanation | 70,71 |
| Increased respiration | Change in membrane processes | 70,71 |
| | pH buffering by ion exchange | 89,90,91 |
| | no explanation | 4,77 |
| Increased adhesion of active cells | | 36,42,43,56 60,87,102 |
| Higher activity of attached cells | | 8,24,44,50 |
| | no explanation | 54,81,83 |
| Decreased substrate utilization | Desorption limitation | 17,20,31,66, 93,94,99,90 |
| | Diffusion limitation | 53 |
| | Lower substrate concentration | 38 |
| | no explanation | 1,30 |
| Lower substrate affinity | Diffusion limitation | 9,40 |
| Change in pH optimum | Proton concentration at surface is different from the bulk | 38,39 |
| Difference in fermentation | Surface is electron acceptor. | 69 |
| Increase in productivity | Immobilization of biomass. | 67,68,97 |
| Decreased mortality | Decreased phagocytosis | 34,47,98 |
| | Other. | 10,16 |
| No effect | | 30,35,70 74,75,85 |

bacteria, substrates, sterility etc.. The relevant but diverse literature is summarized in table 1 together with explanations made by the authors for the outcome of their experiments. From this table it is obvious that no general line can be drawn from the effects reported; on the contrary even opposite effects are described.

It is the objective of this paper to critically review the relevant literature in order to extract a realistic picture of the influence of interfaces on microbial activity. In this review we are mostly interested in the interaction between cells and solid surfaces, and not between cells mutually, as e.g. in biofilms. Therefore, we will limit ourselves to literature dealing with microbes adhering, at most, as a monolayer of cells on inert solid surfaces. Special attention will be payed to results from laboratory experiments.

## 7.2 MECHANISMS OF ADHESION

Bacterial adhesion can be described as a four-step process (Chapter 1):

(i) Diffusive or convective transport of cells to the surface.
(ii) Initial adhesion which is physicochemical in nature, and usually reversible (61).
(iii) Permanent attachment by e.g. polymer bridging (28).
(iv) Multiplication of cells and formation of a biofilm.

For short term laboratory experiments the initial adhesion is most important, therefore we will discuss this step in more detail:

A bacterial suspension is a colloidal system, and adhesion can in a first approximation be described by colloid chemical theories, e.g. the DLVO theory (61). This theory describes the change in the Gibbs energy as a function of separation distance between two surfaces. The total interaction Gibbs energy is obtained from the summation of the Van der Waals interaction and the electrostatic interaction. The latter is for biological systems usually repulsive.

According to the DLVO theory three situations are possible (see Chapter 2, Fig. 4):

(i) a repulsion between the bacterium and the surface when the electrostatic interactions are dominating,

(ii) a strong (irreversible) attraction when Van der Waals forces are dominating,

(iii) a weak (often reversible) attraction in intermediate cases.

In case (iii) adhesion takes place at a certain separation distance from the surface, in the so-called secondary minimum(61). Recently, the occurence of secondary minimum adhesion has been confirmed by interference reflection microscopy (27). The distance between cell and glass surface depended on the cationic composition, as can be expected for secondary minimum adhesion.

Initial bacterial adhesion generally is a reversible process (11,61). This implies no direct contact between cell and surface, and a continuous exchange between the free and adhered populations, making it difficult to distinguish between the activity of adhering and free cells. Hermansson and Marshall (44) experimentally showed this exchange between free and adhered cells, whereby the exchange decreased with an increase of bacterial adhesion strength. The exchange of cells is enhanced in mixed systems due to convective transport of cells (61,82). In order to prevent exchange between surface and suspended population, or even rule out the suspended population, cells have to be irreversibly attached to the surface (35,61,74). A second problem arising from the reversibility of adhesion is the estimation of the total biomass, because a fraction of the cells will always remain adhered.

Adhesion of microbes generally is promoted during exponential growth because of an increased cell hydrophobicity during this growth stage (23,87,102, Chapter 4). This fact makes surfaces a selective locus for metabolically active bacteria. Thus, the finding that in a natural population of microorganisms attached bacteria are more active than free cells (36,42,49,50,56,81), is not necessarily provoked by an activity stimulating effect of surfaces.

In conclusion the following statements can be made on the influence of adhesion on bacterial activity measurements:

- In the case of reversible adhesion it is difficult to distinguish between free and adhered cells.
- Active cells have in general better adhesion properties then resting cells.

## 7.3 ADSORBED SUBSTRATES

Certain compounds or nutrients may accumulate at the interface, thus making interfaces different from the bulk medium. A positive influence of surfaces on bacterial activity is often attributed to the accumulation of nutrients at the surface (8,25,46,101). The increased nutrient and substrate concentration is thought to stimulate bacterial growth rate or increase the yield. The energy from a reaction available for work, in this case biomass production, is called the Gibbs energy. The net reaction Gibbs energy is, at constant pressure and temperature, depending on the chemical potential ($\mu$) of the reactants. If there is equilibrium between the adsorbed and dissolved substrate, the chemical potential is in both phases identical (Fig. 1). This means that the net Gibbs energy resulting from conversion will be independent from the state of the compound; i.e. the cell yield will be independent whether adsorbed or free substrate molecules are used, provided that the metabolic processes remain identical for adsorbed and free bacteria.

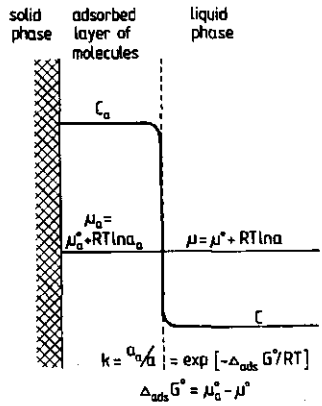
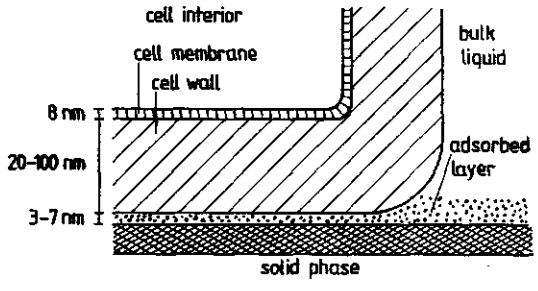



Figure 1 Schematical profile of the chemical potential ($\mu$) and concentration of an adsorbing compound.
K: adsorption constant,
$\Delta_{ads}G^\circ$: adsorption Gibbs energy,
R: gas constant,
T: absolute temperature.

For a good interpretation of the observed effects one must be aware of the dimensions in bacterial adhesion (Fig. 2). Bacteria are 1-2 $\mu$m in diameter, and have a cell wall of 20-100 nm thickness. Reversible adhering cells are at a distance (approx.

5 nm) from the surface due to the electrostatic repulsion between cells and surface (61). The thickness of an adsorbed organic layer usually does not exceed a few nanometer, which implies that only a very small part of the bacterial surface is in direct contact with adsorbed substrates. The cell will predominantly use dissolved (or desorbed) substrates. Their concentration will determine the bioconversion rate. Therefore, adsorption of substrates may result in a decreased bioconversion rate. Adsorption of inhibitors or toxic compounds may on the other hand stimulate bacterial activity.

In a system where the conversion of substrates is desorption limited, adhered bacteria can probably profit from their position near the substrate. At first the diffusion distance of the substrate to adhered cells is shorter then to suspended cells. Secondly, due to the conversion of substrate by adhering micro-organisms the substrate concentration gradient near the surface will become steeper, which results in a faster desorption, and thus conversion, of substrates (Fig. 3).



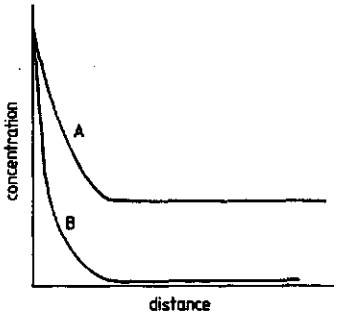




Figure 2 Schematic representation of an adhering cell.

Figure 3 Concentration gradient near a surface without (A) and with (B) substrate conversion by attached bacteria.

In conclusion we can state that substrate adsorption can result in:

- decreased bacterial activity, due to a decreased concentration of nutrients in the medium,
- increased bacterial activity due to a decreased concentration of inhibitors or toxic compounds,
- increased activity of adhered cells when growth occurs mainly on desorbed substrate.

## 7.4 MICROBIAL GROWTH ON INERT SOLID SURFACES

When bacteria are adhered to a surface and start to grow several growth patterns may arise, depending on the mode of attachment:

(i) Cells are reversibly adhered to the surface and each other. This will result in a constant equilibrium between cells at the surface and in the bulk.

(ii) Cells are irreversible bound to the surface (by e.g. polymers) but not to each other, resulting in the formation of a complete monolayer of cells on the surface.

(iii) Cells are irreversible attached to the surface and each other, resulting in biofilm formation.

In case reversible adhering cells divide, newly formed cells may initially remain attached, however, the amount of adhering cells will tend to remain in equilibrium with the concentration of suspended cells. If detachment is a relatively slow process (61), small microcolonies may develop, as observed by Caldwell (12,13) for the growth of *Pseudomonas fluorescens* on glass surfaces.

Growth kinetics of adhered bacteria can best be compared to batch growth kinetics including a term for the netto at- or de-tachment rate (A, expressed in: cells.$m^{-2}$.$h^{-1}$). This last term is influenced by the adhesion characteristics and the transport of cells from or to the surface:

$$dX_a/dt = \mu_a X_a + A$$

Where $X_a$ is the amount of cells on the surface (cells.$m^{-2}$) and $\mu_a$ is the specific growth rate of adhered cells ($h^{-1}$). Thus, in a batch culture growth kinetics on surfaces differ not very much from growth kinetics in the bulk phase. When the growth rate of adhered and suspended cells is different, the occurence of surface growth can nevertheless be inferred from the growth curve of the suspended population (Fig. 4).

As compared to batch cultures in continuous culture growth kinetics on a surface can deviate from the bulk phase; since attached cells are only removed by detachment, surface growth is more or less uncoupled from the dilution rate. Surface growth in fermentors results in an increased productivity (DX) especially at high dilution rates. Above the maximal dilution rate (maximal

growth rate of a bacterium) the microbes remain in the fermentor (3,59,80,97, Fig. 5). A high amount of bacteria on surfaces can function as a buffer, flattening of the effects of changes in dilution rate (53).

In the following paragraphs the foregoing mainly theoretical discussion will be used to discuss and interprete literature data on the possible influence of surfaces on microbial activities. This is done in order to come to general conclusions how adhesion may influence bacterial activities. The discussion will be ordered in three separate paragraphs:

- Direct effects of adhesion on bacterial activities.
- Use of adsorbed substrate by bacteria.
- Indirect effects of adhesion on bacterial activities.

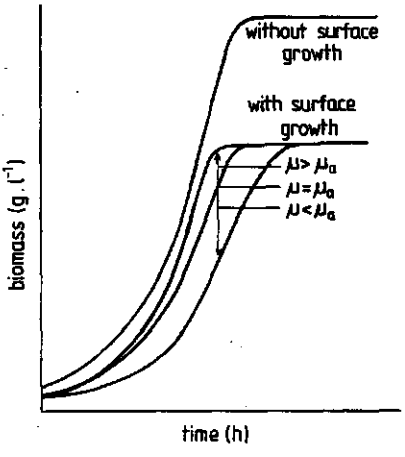



Figure 4 Influence of surface growth on the increase in suspended biomass in a batch culture, for the case of reversible bacterial adhesion.

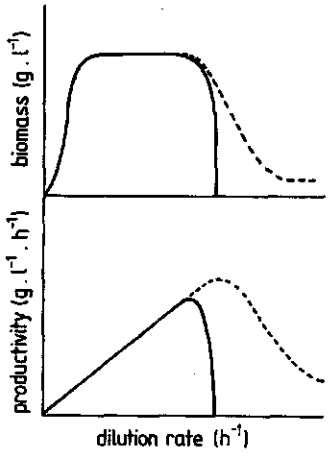



Figure 5 Growth and productivity in a chemostat with (------) and without (———) surface growth.

## 7.5 DIRECT EFFECTS OF ADHESION ON BACTERIAL ACTIVITY

During the 1984 Dahlem workshop on microbial adhesion and aggregation, the discussion group on activity on surfaces concluded: "Attachment to a surface can undoubtedly affect the activity of microorganisms, although sometimes in ways that are

not readily predictable on our current knowledge". This statement still holds, especially in the context of possible direct effects of surfaces on bacterial physiology. Although, there are many experiments performed which suggest a direct influence of adhesion on microbial activity, only one theory is proposed to explain these observations (19). The merits of this theory, which is based on the chemiosmotic theory, will be discussed first. Then experimental evidence for a direct influence of solid surfaces on microbes will be reviewed. This review will be restricted to experiments performed under reasonably defined conditions, and with substrates that do not have a strong interaction with the solid surface. Because of the great diversity in experimental set-up, the experiments are discussed one by one. At the end of this paragraph some conclusions will be drawn.

### 7.5.1 Influence of adhesion on chemiosmosis

Ellwood et al.(19) have speculated on a mechanism to explain why adhesion is beneficial to bacterial activity. They proposed that the proton motive force is positively influenced by the presence of a surface. It is suggested that normally a small fraction of the protons at the outside of the membrane leak away into the medium. The presence of a solid surface will partly prevent diffusion of protons away from the bacterium, and as a consequence (i) increase the efficiency of the membrane processes and (ii) create a more energized bacterial membrane in the vicinity of the solid surface. However, any effect of retardation of proton diffusion will for several reasons be negligible. Firstly, the loss of energy due to leakage of protons is probably negligible. The production of 1 g of cells costs approximatly 0.1 Mol ATP. If this amount of ATP is generated totally by the proton motive force 0.25 Mol of hydrogen ions have to be circulated over the cell membrane. Even a loss of 1 % of the hydrogen ions will give a tremendous shift in the medium pH (to ± 2.5), which is normally not observed. Secondly, loss of protons by microorganisms to the medium has, for sake of electroneutrality, to be compensated by other cations thus keeping the electrostatic potential across the membrane unaffected. Thirdly, the creation of a more energized part of the cell membrane due to the presence of a surface is

not likely, because protons diffuse very easily through the periplasmatic space.

From the foregoing we have to conclude that the theory of Ellwood et al. (19) is not suitable to explain any signifficant changes in bacterial activity upon adhesion.

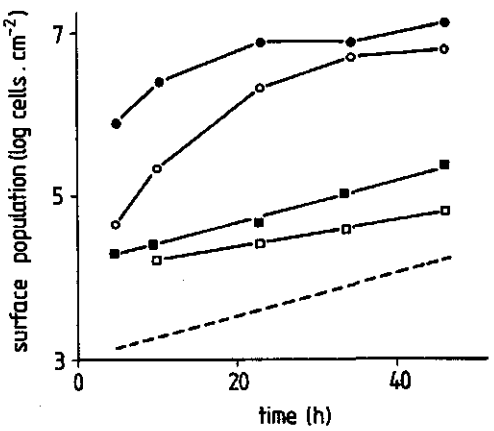



Figure 6 Increase in surface population density of a *Pseudomonas* sp. grown in a carbon limited chemostat (D = 0.06 $h^{-1}$). 50 nM, 500nM, 50 µM, and 5mM glycerol.

------- growth of suspended population.
After Ellwood et al (19).

### 7.5.2 Metabolic activities of adhering bacteria

The above discussed theory was postulated by Ellwood et al. (19) to explain results of a continuous culture experiment in which the colonization of glass surfaces by a *Pseudomonas* sp. was studied (Fig. 6). It was concluded that, at least initially, growth of adhered cells is faster than of suspended cells. The authors suggested that the increased rise in surface population density can only for a small part be due to adhesion of suspended cells. However, another explanation for these observations may be that in a chemostat surface growth is uncoupled from the dilution rate (Par. 7.4). Therefore as compared to the suspended population, the adhered population has the possibility to increase its size more rapidly until a certain equilibrium situation is reached (in the discussed case after about 20h).

Table 2 Relative product formation from glucose by *E. coli* as reported by Morisaki (69).

| | lactic acid | succinic acid | ethanol | acetate | $CO_2$ |
|---|---|---|---|---|---|
| without resin | 57% | 13% | 13% | 13% | 4% |
| with resin | 35% | 0% | 13% | 13% | 39% |

The influence of adhesion on bacterial activity has been extensively studied by Fletcher et al. (8,9,24,25,26). The results of these studies indicate that there is no general "surface effect", and the effect of surfaces on activity depends heavily on environmental conditions and substratum properties. A direct and strong positive influence of adhesion has been shown in only one article (26). Here, the glucose assimilation by adhered cells exceeded that of free-living cells by a factor of 2 to 5 or even more. Respiration of glucose by surface associated cells was greater than by free-living bacteria (26). However, these results are probably an experimental artefact. From the data in the article it can be calculated that during the two hours of incubation of suspended cells, glucose is totally consumed (probably already within 30 minutes), whereas this is not the case in the incubation of adhered cells. e.g. From fig. 1 in the ref. 26 it can be calculated that when 28 µg C is incubated the total consumption during the incubation of suspended cells was 50 µg C against 4.4 µg C for the adhered cells.

Addition of an ionexchange resin has been shown to induce several changes in bacterial activity (38,39,40,41,69): (i) a decreased substrate oxidation (38), (ii) a shift in the pH optimum to higher pH with an anionic resin and to lower pH with a cationic resin (38), (iii) a shift in the fermentation of glucose to more oxidized end products (table 2, 69). The first two observations were made with a system of 1 g of resin (in the $Cl^-$-form) per ml of 0.07 M phosphate buffer. This high amount of resin may give mixing problems (i.e. substrate or oxygen diffusion limitation) and a shift in pH due to an exchange of chloride ions against phosphate or hydroxy ions. The existence of a cationic layer, as suggested by the authors to explain the shift in optimum pH (38), is in physical-chemical terms unrealistic. The shift in glucose fermentation products (Table 2, 69) may, since a mass balance is absent, also be due to the fact that the anionic compounds such as succinate and lactate bind to the anion exchange resin.

An increased respiration and decreased glucose consumption rate in the presence of a $C_{12}$ or $C_{13}$ alkane/water interface has been reported by Morisaki (71). However with several other alkanes

this effect has not been observed. A similar effect has been observed upon addition of three different solids, with nine other solids no effect was observed (70). As only a very small fraction of bacteria was associated with the interface, a direct effect from adhesion is in this case not likely. The observations might be explained as an uncoupling effect on the electron transport chain by the alkanes or impurities in the commercial materials. Humphrey and Marshall (48) showed that a surfactant-like impurity in dialysis membranes had a similar effect as reported by Morisaki.

A 25% increased growth rate and a broader pH range for *Nitrobacter* cells attached to glass, has been described by Keen and Prosser (52). They reasoned that their observation is not due to a concentration of nutrients or an altered pH at the surface but more likely the result of an extracellularly slime layer formed by attached cells. This layer aids to create a micro-environment low in nitrite concentration (which is the substrate of Nitrobacters but shows also toxic effects).

Many experiments on the relation between bacteria and solid surfaces are performed with clay minerals as the solid phase. Filip (22) and Stotzky (92) showed that in these cases it is not possible to directly relate observed changes in bacterial activity to adhesion, because addition of clays to a bacterial suspension promoted growth irrespective whether the clay was applied directly to the solution or in a dialysis bag. The indirect influence of clay minerals on bacterial activity will be discussed later.

Finally we want to point to the fact that in several articles no (direct) effect of solid surfaces has been reported (Table 1). For instance, Gordon et al. (30) used microcalcrimetry and respirometry to detect changes in activity upon adhesion of *Vibrio alginolyticus* to hydroxyapatite. They showed that bacterial activity (i.e. heat and $CO_2$ production), on glucose or glutamate was not enhanced by the presence of particles, regardless whether the bacteria, the organic nutrient, or both were associated with the surface. Also other authors report no signifficant difference in the specific activity of irreversible attached *Saccharomyces cerevisae* (ethanol production from glucose) or *Arthrobacter simplex* (prednisolone production from cortisol) cells (35,74,85).

### 7.5.3 Substrate affinity of adhered cells

A decrease in substrate affinity, or increase in $K_s$, is regularly reported (9,13,40). According to Bright and Fletcher (9) there are two possible explanations for the difference in $K_s$ between free and adhered cells: (i) the difference could be a system property (i.e. diffusion limitation) or (ii) the higher $K_s$ values for surface-associated cells is a reflection of a real difference in assimilation behaviour. The former explanation is probably the most realistic since the change in $K_s$ is independent of the type of attachment surface (9). The determination of "apparent" $K_s$ values is actually used to determine substrate diffusion limitations of adhered cells. Moreover, Caldwell (13) showed a glucose diffusion limited growth of adhered cells, even at glucose concentrations of 100 mg/l. The decrease in growth at high surface population density as observed by Ellwood (19) might also be caused by substrate diffusion limitation.

Jeffrey and Paul (51) suggested from activity measurements on attached and free living *Vibrio* sp. that not only the apparent substrate affinity but also the maximal substrate conversion rate of attached cells can change. The latter is due to the fact that part of the cell surface (± 20%) is unavailable for substrate uptake.

### 7.5.4 Conclusion

As a conclusion of this section we can state that there is no clearcut evidence at all that bacteria are directly influenced by adhesion. Effects that in the literature are ascribed to adhesion can, in most cases, be explained in another theoretical more sound way. In a system where adhered bacteria are growing on a soluble substrate, substrate diffusion to the cells may become rate limiting. This results in a higher substrate affinity constant for adhered cells.

## 7.6 USE OF ADSORBED SUBSTRATES BY BACTERIA

### 7.6.1 Growth on small molecules

Classical examples for the positive influence of solid surfaces on bacterial activity are the experiments described by Heukelekian and Heller (46) and Zobell (101). These authors stated that solid surfaces are stimulating growth especially at low nutrient concentrations (< 10 mg/l). As indicated in paragraph 7.2 this cannot directly be explained by adsorption of nutrients on the surface.

Heukelekian and Heller (46) observed the growth of *E. coli* in a glucose/peptone medium with and without glass beads. Growth of *E. coli* was stimulated in the presence of glass surfaces, especially at low nutrient concentrations (Fig. 7). The growth stimulation is even greater than indicated in Fig. 7 since a part of the cells were adhered to the glass surface, and thus were not accounted for in the measurement of the total viable count. The experiment of Heukelekian and Heller is often referred to but never confirmed in the literature. Therefore, we have repeated this experiment as good as was possible from the original description. All our glassware was cleaned in chromic-sulfuric acid, and afterwards rinsed several times with destilated water. Our results are also given in Fig. 7. The experiment was repeated three times and always a decrease in cell yield in the presence of glass beads was found. The same has also been observed by Jannash (49). Adhered cells are not accounted for and therefore the aparent growth yield in flasks with glass beads is lower. The discrepancy between our results and those of Heukelekian and Heller is difficult to explain. One possibility might be the presence of a small amount of organic carbon on the glass beads in the experiments of Heukelekian and Heller. Unfortunately, it is impossible to draw any clearcut

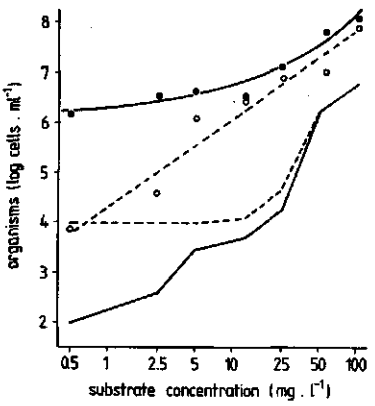



Figure 7 Growth of *E. coli* on glucose/pepton medium. with (------) or without glass (——) beads. Lines with data points are own measurements, the other are taken from ref. 46.

conclusion since the cleaning procedure has not been described in the original article. A small fraction of organic material on the glass beads can also explain why at low substrate concentrations (between 12.5 and 0.5 mg/L) no change in total cell yield is observed, which otherwise is difficult to substantiate. Another remarkable point in the results of Heukelekian and Heller is the strong dependence of cell yield on the added amount of substrate. If the substrate concentration is decreased with a factor 10 (from 100 to 10 mg/L) the cell yield decreases with a factor 100. Alltogether we feel that on the basis of this experiment it is not very likely that a pure "surface-effect" exists.

Introduction of a solid phase in a liquid medium decreases the concentration in solution of compounds that adsorb at the liquid/solid interface. When a non-inhibiting substrate is adsorbed this may result in a decreased free substrate concentration and thus a lower substrate utilization rate. Examples are the reduction of ammonium oxidation in the presence of different clay minerals (29) and the reduction of succinate assimilation in the presence of an anionic resin (38). Moreover, Ogram et al. (79) showed that in a soil slurry with (2,4-dichlorophenoxy)-acetic acid only substrate in solution is degraded by the attached and suspended bacteria. This indicates that the substrate must first desorb before degradation can take place.

A decreased substrate concentration due to adsorption can, in the case of toxic substrates, lead to an increased microbial activity. The degradation rate of e.g. benzylamines becomes at low benzylamine concentrations (0.02 to 200 µg/l) smaller upon addition of montmorillonite, whereas at high concentrations (20 mg/l) degradation was enhanced in the presence of montmorillonite (94). Addition of activated carbon has been shown to protect microorganisms from toxic levels of phenol (up to at least 17 g/l), and therefore stimulating its conversion (18,72). The same has been observed for the degradation of aldehydes in the presence of montmorillonite (92). Adsorption of inhibitors on surfaces and their consequent removal from the solution has been shown to promote bacterial growth (37,95).

With strongly adsorbing (mostly hydrophobic) substrates, biodegradation can become desorption limited. In this case

bioconversion will be dependent on the solid surface area. Thomas et al. (96) conducted a study of the relationship between the dissolution rates of organic compounds that are sparingly soluble in water and the biodegradation of these compounds. Bacterial growth caused a decline in the concentration of naphtalene or 4-chlorobiphenyl in solution. When the compounds were no longer detectable in solution, the bacteria stopped growing. Similar results have been found for the bioconversion of hexachlorocyclohexane (86) and n-alkylamines (100). Although desorption seemed in all these studies to become rate limiting for biodegradation, the biodegradation rate was still greater then the rate of desorption in sterile systems. Seemingly, the actual desorption rates in sterile systems differ from those in non-sterile systems. This may be due to an increased concentration gradient near the surface as a consequence of microbial activity (see paragraph 7.3).

In case of irreversible adsorption the compounds seem to be protected against microbial attack. This has been observed for aspartate, cysteine (17), diquat (99), and several proteines (92) adsorbed onto montmorillonite. In table 3 we have summarized the literature related to bioconversion of adsorbed substrates.

### 7.6.2 Bacterial growth on macromolecules

Zobell incubated non-sterile seawater in glass bottles with different surface/volume ratios. In bottles with high surface to volume ratio's the greatest oxygen consumption and the greatest increase in bacterial counts was measured. The oxygen consumption (< 30 nM/L) and organic carbon consumption (< 0.9 mg/L) were very small. A minor organic impurity (e.g. on the glass surface) can thus have a great influence on the results, especially at high surface to volume ratio's. Zobell has tried to prevent organic contamination by cleaning all his glass-ware in hot chromic-sulfuric acid. He explained the obtained results as follows: "It is believed that besides concentrating nutrients by adsorption and providing a resting place for sessile bacteria, solid surfaces retard the diffusion of exoenzymes and hydrolyzates away from the cell thereby promoting the assimilation of nutrients which must be hydrolyzed extracellularly prior to ingestion".

Table 3 Literature related to conversion of adsorbed substrates

| Substrate | Surface | Reference |
|---|---|---|
| | **Decreased conversion/Desorption limitation** | |
| Amino-acids | Montmorillonite/kaolinite | 17 |
| Proteines | Montmorillonite/silicagel | 20,66,92 |
| Acetate,Succinate Glutamate,Citrate | Hydroxyapatite | 31 |
| Succinic acid | Ion exchange resin | 38 |
| n-Alkylamines | Bentonite | 100 |
| Benzylamine (Low C) | Montmorillonite | 94 |
| Na-Oleaat | Montmorillonite | 92 |
| (2,4 dichlorophenoxy)-acetic acid | Soils | 79 |
| DNA | Montmorillonite | 33 |
| | Sand | 62,63,64 |
| Atrazine,Chlorthiamid | Charcoal,Soil | 73 |
| Pentachlorophenol | Barkchips | 2 |
| n-Eicosane | | 15 |
| Naftalene,4 Cl-Biphenyl Octadecane | | 96 |
| | **Increased conversion** | |
| Phenol | Activated carbon | 18,72 |
| Benzylamines (high C) | Montmorillonite | 94 |
| Aldehydes,Vanillin | Montmorillonite | 92 |
| Proteines | Montmorillonite/Kaolinite | 21,101 |
| | **Inhibited conversion** | |
| Aspartate,Cysteine | Montmorillonite,Kaolinite | 17 |
| Diquat | Montmorillonite | 99 |
| Proteins | Montmorillonite | 92 |

To our knowledge, direct experimental evidence that bacteria profit from surfaces due to a retardation of diffusion of exo-enzymes or hydrolyzates has not yet been published. But this view is supported by an experiment of Hermansson and Dahlbähk (43). They showed that when proteins are spread on the air/liquid interface at a relative high surface coverage, a high amount of labelled $CO_2$ was released in the bulk solution. This is presumably due to diffusion of hydrolyzates from the surface to the bulk liquid. When proteines were applied at low surface coverage all the protein was converted by cells adhered to the air/liquid interface. The presence of surfaces has also been shown to enhance

the bioconversion of caseinate, lignoprotein, chitin, or lysozyme, but not of glucose, glycerol, lactate, and hydrolyzed lysozyme (21,101).

In general degradation of polymers is retarded by the presence of inert surfaces. A decreased degradation was observed for proteines (20,66,92) and for DNA (33,62,64). This decrease could be the result of various factors such as desorption limitation, conformation changes of adsorbed polymers, or adsorption of exoenzymes. An influence of polymer conformation was shown by Marshmann and Marshall (66) who studied bacterial growth on proteines (gelatine, BSA, and lysozyme) in the presence of different amounts of clay minerals (montmorillonite and kaolinite). Depending on the protein-to-clay ratio different effects have been observed. At a high protein to clay ratio, growth was not affected by the clay, at intermediate protein to clay ratio's, growth rate but not final cell yield was reduced, and at low protein to clay ratio's the adsorbed protein was unavailable for hydrolysis. Adhesion of proteins is usually entropically driven, which means that adsorbed proteins usually have an increased fraction of random coil structure. This may make the adsorbed proteins less available for hydrolization.

### 7.6.3 Conclusion

As a summary of this paragraph it can be said that solid surfaces influence substrate utilization by decreasing the free substrate concentration. In the case of toxic or inhibiting compounds an increased activity may be observed; in the case the substrates are not toxic the activity may decrease. Irreversible adsorption of a substrate prevents its bioconversion. In some cases desorption of substrates may be rate limiting for bioconversion.

Table 4 Indirect effects of the presence of a solid phase on microbial activity

| Effect | References |
|---|---|
| pH buffering | 14,58,89 90,91 |
| Protection against dessication | 10,65 |
| virusses | 49,84 |
| protozoa | 34,47,98 |
| chlorination | 16,45 |
| radiation | 6,76 |
| Increased productivity at high dilution rates | 3,12,59,67 68,97 |
| Increased DNA transformation | 64 |

## 7.7 INDIRECT EFFECTS OF SURFACES ON BACTERIAL ACTIVITY

Surfaces can have various indirect influences on bacterial activity (Table 4) due to a modification of the physico-chemical environment of the microbes, or the interaction between a microbe and its surrounding. The influence of clay minerals is in this context extensively studied, especially by Stotzky (92). From some 100 samples of clay minerals and various particles which posses some of the characteristics of clays, essentially only samples of montmorillonite stimulated the respiration of bacteria, primarily by maintaining the pH of the environment suitable for sustained growth. This was confirmed with more than 20 bacterial species differing in morphology, motility, Gram reaction, stage of growth etc. (89,90,91,92). The maintenance of a favorable pH was found to be dependent on the initial pH of the system, the relative basicity of the cations on the clay, and the buffer capacity of the clay particles.

Survival of bacteria in soils has also been found to be related to the presence of, especially montmorillonite-like, clay minerals (65). These clays can form a coating on the bacterial cell surface. Consequently, such a coating may protect bacteria against adverse environmental conditions (10), protozoa grazing (47) or virus attack (49). The latter can also be prevented immobilizing virusses on clay (34). The type of protection against viruses was found to depend on the ionic strength. At low ionic strength bacteria

bacteria are protected against virusses by a clay envelope, at high ionic strength, however, the virusses themselves adhere strongly to soil or sediment particles (84). A clay envelope can also protect bacteria from dessication. Bushby and Marshall (10) found that the resistance of fast growing *Rhizobia* to dessication was related to the presence of montmorillonite. From an examination of water adsorption isotherms it was suggested that susceptibility to dessication is related to the relative high state of internal hydration at low vapor pressures. As montmorillonite has a higher affinity for water, the existence of a clay envelope at the bacterial surface may protect the cells by reducing their internal hydration status to a level where most enzyme activity ceases.

Another mode of promotion of survival of attached bacteria can be the embedding of attached cells in polymeric matrices or "slimes". These highly hydrated, and frequently charged gel forming polymers (usually polysaccharides) may protect cells from potential toxic effects by complexing heavy metals, retarding diffusion of inhibitors, or by resisting desiccation (28,92,52).

There are several other indirect effects of surfaces mentioned in the literature wich are summarized below.

High clay concentrations in liquid media (4% montmorillonite or 40% kaolinite) have an inhibitory effect on bacterial activities, probably due to an increased viscosity which retards the diffusion of oxygen to the cells (92).

In systems with high dilution rate's (e.g. streams, the mouth, continuous cultures), the productivity can be increased due to the presence of an adhered population, in particular near or above the critical wash-out rate (3,12,59,67,68,97).

Lorenz et al. demonstrated that adsorbed DNA is protected from degradation by DNA-ase (62, 63). Also the DNA of dead cells was found to be relatively stable in the presence of solids (33,78). Transformation of DNA to *Bacillus subtilis* in the presence of sand grains has been found to be, compared to liquid cultures, 25 - 50 times increased (64). Although the precise mechanisms behind these observations are not known, they indicate that solid/liquid interfaces may stimulate transformation of DNA, and therefore the spread of DNA throughout a bacterial community.

## 7.8 GENERAL CONCLUSIONS

In many experimental systems it has been observed that solid surfaces either stimulate, inhibit, or have no effect on bacterial activities. The results often depend on the organism, kind and concentration of substrate, and of the type of solid surface. On the basis of this review we can state that there is no experimental or theoretical evidence that adhesion directly influence bacterial metabolism. Differences in activities of adhered cells, as compared to free cells, can be explained by:

- better adhesion properties of active cells,
- a limiting substrate diffusion from the bulk liquid to the surface,
- a benifit from the use of desorbed substrate by adhered cells,
- a benefit due to retardation of diffusion of exoenzymes and hydrolyses products produced from macromolecules at a position close to the adhered bacterial cells,
- a decreased concentration of nutrients in the bulk which generally results in a decreased activity but in the case of toxic compounds leads to an increased activity,
- irreversible adsorption of the substrate which generally inhibits bioconversion.

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## SUMMARY AND CONCLUDING REMARKS

As mentioned in the introduction of this thesis bacterial adhesion has been studied from a variety of (mostly practice oriented) starting points. This has resulted in a range of widely divergent approaches. In order to elucidate general principles in bacterial adhesion phenomena, we felt it was necessary to start from a fundamental level i.e. using well-defined model systems. In our study colloid chemical principles are applied to microbial systems. Although both colloid chemists and microbiologists have investigated the behaviour of small microscopic particles, there has been only limited cooperation between them in the past. Nevertheless, this study reveals that such a cooperation can be very fruitful.

After a general (Chapter 1) and a theoretical (Chapter 2) introduction, we deal in Chapters 3 and 4 with the relation between bacterial surface characteristics and adhesion to sulphated polystyrene (a hydrophobic, charged surface). The cell surface hydrophobicity and electrokinetic potential were determined by the contact angle measurement and electrophoresis, respectively. Adhesion increases with increasing bacterial hydrophobicity or decreasing electrokinetic potential. The effect of the electrokinetic potential increases with decreasing hydrophobicity. An interesting finding is the increase with growth rate in surface hydrophobicity of bacteria.

In Chapter 5 we show that initial adhesion to sulphated polystyrene is reversible and can at least qualitatively be described by the DLVO theory for colloidal stability, i.e., in terms of Van der Waals and electrostatic interactions. From adhesion isotherms we found an adhesion Gibbs energy of -2 to -3 kT per cell. This corresponds to calculations using DLVO theory that predict adhesion in the so-called secondary minimum, a case where no direct intimate contact is made between bacterium and surface. Finally, the implications of our findings for natural and (bio)technical processes are discussed.

In Chapter 6 we report on the applicability of the DLVO theory for the interpretation of bacterial adhesion to glass and to more practical surfaces (Rhine river sediment and protein-coated surfaces). In all these cases adhesion could be interpreted in terms of the hydrophobicity and electrical properties of the surfaces.

The possible influences of adhesion on bacterial activity are discussed in Chapter 7, in the form of a critical literature review. Despite the opinion regularly heard that there might be a direct influence of adhesion on bacterial physiology we have not been able to find any experimental evidence in support of this hypothesis. Different activities of attached and free cells are often due to changes in substrate transport (e.g. diffusion, desorption, or convective transport) or differences in hydrophobicity of active and resting cells. For the conversion of adsorbed substrates the dissolved concentration determines the conversion rate. With strongly adsorbing compounds the conversion can become desorption-limited, whereas non-desorbing compounds are often not degraded.

In this thesis it is shown that application of colloid chemistry to microbial systems can lead to interesting new viewpoints. More specifically, the DLVO theory for colloidal stability was found to give a quantitative description of the initial stage of bacterial adhesion both to model surfaces as in more applied situations (Chapters 5 and 6). Generally, in the studies dealing with interaction between bacteria themselves or between bacteria and surfaces electrostatic interactions are often neglected, despite the fact that this interaction is often desicive whether strong adhesion can occur or not.

The insights derived from a colloid chemical approach can be used, as complementary to a more biological approach, in understanding the (auto-)immobilization of bacteria in natural and biotechnological systems, as e.g. in UASB-reactors.

The experimental methods developed in this study may also be successfully applicable in other research areas. Due to the sensitivity of the contact angle and electrophoretic mobility measurements they can for instance be applied as a rapid screening

method of new isolates or cell surface mutants. Especially with surface mutants the methods mentioned here are much faster than conventional biochemical or immunological methods.

The contact angle and electrophoretic mobility measurements may also be useful for obtaining information on the structure of the outer part of the cell wall. In particular electrophoresis, at different pH and electrolyte strength, combined with chemical modifications of specific groups (e.g. $-NH_2$ groups) may be very powerful. Preliminary experiments with lipopolysaccharide mutants of Pseudomonads are very promising. For this and other applications it is necessary to improve the electrochemical characterization of bacteria, especially with respect to the influence of bacterial conductivity.

Other areas in microbiology that may be successfully treated by colloid chemical theories concern firstly the biological availability of substances, in particular micro-pollutants, to bacteria. This availability is mainly determined by substrate adsorption to inert solid material and substrate transport through the cell wall and membrane. A second interesting field might be the relation between molecular composition and function or stability of membranes in different bacteria, or under different environmental conditions.

## SAMENVATTING EN SLOTOPMERKINGEN

Zoals reeds in de introductie van dit proefschrift is vermeld wordt bacteriële adhesie tot nog toe voornamelijk bestudeerd vanuit een praktijk gericht gezichtspunt. Dit heeft geresulteerd in een reeks uiteenlopende benaderingen. Om algemene principes op te helderen hebben we daarom voor een meer fundamentele aanpak gekozen. Hiertoe hebben we colloïd-chemische principes toegepast op microbiologische systemen. Alhoewel zowel colloïd-chemici als microbiologen het gedrag van microscopisch kleine deeltjes bestuderen is er in het verleden slechts van een zeer beperkte samenwerking sprake geweest. Dit onderzoek toont echter dat een dergelijke samenwerking bijzonder vruchtbaar kan zijn.

Na een algemene (Hoofdstuk 1) en een theoretische (Hoofdstuk 2) inleiding, wordt in de Hoofdstukken 3 en 4 de relatie tussen bacteriële oppervlakte karateristieken en adhesie aan gesulfateerd polystyreen (een hydrophoob, negatief geladen oppervlak) behandeld. De hydrophobiciteit van het celoppervlak en de elektrokinetische potentiaal zijn respectievelijk bepaald via de randhoek van een druppel water op een laag cellen en electroforese. De adhesie neemt toe met toenemende hydrophobiciteit en afnemende electrokinetische potentiaal. Het effect van de electrokinetische potentiaal neemt toe met afnemende celwand hydrofobiciteit. Een interessante constatering was de toename in hydrofobiciteit met de groeisnelheid van bacteriën, hetgeen met name een ecologische betekenis kan hebben.

In Hoofdstuk 5 wordt getoond dat initiële adhesie aan gesulfateerd polystyreen reversibel is en op zijn minst kwalitatief goed beschreven wordt door de DLVO theorie voor kolloidale stabiliteit, dat wil zeggen in termen van Van der Waals en electrostatische wisselwerkingen. Uit adhesie isotermen kan een adhesie Gibbs energie van -2 tot -3 kT per cel worden berekend, hetgeen goed overeen komt met berekeningen volgens de DLVO theorie voor adhesie in het zogenaamde secundaire minimum. Het Hoofdstuk wordt afgesloten met een discussie over de betekenis van onze bevindingen voor de beschrijving van adhesie processen in natuurlijke en (bio)technologische systemen.

De toepasbaarheid van de DLVO theorie voor de beschrijving van adhesie aan glas en meer praktische oppervlakken (Rijn sediment en met eiwit bedekte oppervlakten) wordt in Hoofdstuk 6 behandeld. In al deze gevallen kon de adhesie worden geïnterpreteerd aan de hand van de hydrophobiciteit en electrokinetische eigenschappen van de oppervlakken.

In Hoofdstuk 7 wordt, middels een kritisch literatuur overzicht, een discussie gegeven over de mogelijke beïnvloeding van de bacteriële activiteit als gevolg van adhesie. Ondanks de regelmatig gehoorde opinie dat er een directe invloed is van adhesie op de bacteriële fysiologie, hebben we in de literatuur hiervoor geen enkel experimenteel bewijs gevonden. Verschillen in activiteit tussen vrije en gehechte cellen zijn vaak het gevolg van verschillen in substraat transport (door diffusie, desorptie, of convectief transport) of een verschil in hydrophobiciteit (dus adhesie) tussen actieve en niet actieve cellen. Voor de omzetting van geadsorbeerde substraten is de opgeloste concentratie snelheidsbepalend. De omzetting van sterk adsorberende substraten kan desorptie bepaald zijn, terwijl irreversibel geadsorbeerde substraten niet meer beschikbaar zijn voor de bacteriën.

In dit onderzoek is getoond dat toepassing van de colloïd chemie op microbiologische systemen kan leiden tot nieuwe interessante gezichtspunten. In dit geval is gebleken dat de eerste stap(pen) van de bacteriële adhesie in eerste benadering vanuit de DLVO theorie kunnen worden beschreven. Over het algemeen wordt in dit verband de invloed van de electrostatische wisselwerking vaak vergeten. Deze wisselwerking blijkt echter wel bepalend voor de vraag of reversibele dan wel irreversibele adhesie optreedt.

De inzichten verkregen uit een colloïdchemische benadering van adhesie kunnen, samen met een meer biologische benadering, worden gebruikt bij het onderzoek naar immobilisatie van microorganismen in natuurlijke en (bio)technologische systemen. Een dergelijke benadering wordt momenteel reeds toegepast in het onderzoek naar de mechanismen van korrelvorming in zgn. UASB reactoren.

De methodes voor fysische oppervlakte karakterisering zoals in dit onderzoek voor bacteriën ontwikkeld kunnen eveneens succesvol worden toegepast in andere onderzoeksgebieden. Vanwege de gevoeligheid van de randhoek en electroforese metingen, kunnen ze bijvoorbeeld worden gebruikt voor een snelle screening van nieuwe isolaten of celwand mutanten. Speciaal in dit laatste geval wordt veel sneller resultaat verkregen dan met conventionele chemische of immunologische technieken.

Randhoek en electroforese metingen kunnen wellicht ook goede technieken zijn voor onderzoek naar de ruimtelijke structuur van de celwand. Met name electroforese van bacteriën bij verschillende pH's en ionsterkten, gecombineerde met gerichte veranderingen van celwand componenten zou zeer succesvol kunnen blijken. Experimenten met lipopolysaccharide mutanten van Pseudomonaden waren in dit verband veelbelovend. Voor deze en andere toepassingen is het echter noodzakelijk om de electrochemische karakterisering van bacteriën te verbeteren, met name met betrekking tot de invloed van de bacteriële geleidbaarheid op de electroforese.

Een ander microbiologische onderzoeksgebied dat succesvol vanuit de colloïdchemie kan worden benaderd is de beschikbaarheid van substraten, met name micro-verontreinigingen, voor bacteriën. Deze beschikbaarheid wordt vooral bepaald door substraat adsorptie aan inerte oppervlakken en transport door de celwand en membraan. Een ander samenwerkingsgebied vormt het onderzoek naar de betrekking tussen moleculaire samenstelling en functie of stabiliteit van membranen in verschillende bacteriën en onder verschillende milieuomstandigheden.

## NAWOORD

De afgelopen jaren heb ik de mogelijkheid gehad om onderzoek te doen op het grensvlak van twee vakgebieden die voordien nauwelijks onderling contact hadden. Dat dit succesvol is verlopen is mede te danken aan het open karakter en belangstelling van de medewerkers van de vakgroepen microbiologie en fysische en kolloïdchemie. Een aantal personen die een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift wil ik hier met name noemen.

- Willem Norde, omdat hij altijd zeer bereidwillig en enthousiast klaar stond om over de opzet en resultaten van de experimenten te discussiëren, en er daarbij zorg voor droeg dat alles ook colloïd chemisch verantwoord bleef.
- Alex Zehnder, die als initiator en stimulator van dit onderzoek, mij de volledige vrijheid heeft gelaten mijn eigen weg te zoeken. Ook zijn inbreng bij het geordend op papier krijgen van mijn resultaten en gedachten was niet bepaald verwaarloosbaar.
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- De doctoraalstudenten, Hennie Bloemhof, Leon Bremer, Cors van de Brink, Willem Oosterberg, Hubert Sengers, en Bert van de Wal, niet alleen vanwege de directe hulp in het lab, maar ook vanwege de noodzaak voor mij om bij (of tengevolge van) de begeleiding steeds kritisch over het werk na te denken en te discussiëren.
- Gedurende mijn aanwezigheid op de vakgroep microbiologie is er veel vernieuwd. Dit heeft mede tot gevolg gehad dat ik met veel verschillende mensen werkruimtes heb gedeeld. Al deze mensen worden bedankt voor de collegialiteit en sfeer die ze schiepen, met name Hans Brons die steeds met mij (of ik met hem?) mee is verhuisd.
- Ans Broersma en Nees Slotboom, hebben in de vorm van respectievelijk tekstverwerking en figuren tekenen een duidelijk zichtbare bijdrage aan dit boekje geleverd.
- Sjaan Gerritsen, wordt hier dan wel als laatste genoemd maar was toch vaak de eerste die ik iedere dag op het lab ontmoette, onder het genot van een kop verse koffie.

Ten slotte wil ik Huub Rijnaarts succes toewensen met zijn onderzoek en de hoop uitspreken dat er nog lang een dermate goede en vruchtbare samenwerking in stand blijft tussen de vakgroepen microbiologie en fysische en kolloïdchemie als tijdens de afgelopen drie jaar.

P.S. Degene die na lezing van dit proefschrift de behoefte voelt het onderhavige onderzoek te karakteriseren als fundamenteel danwel toegepast wordt verzocht eens een andere bril op te zetten.

# CURRICULUM VITAE

Marinus Cornelis Maria van Loosdrecht werd op 15 juli 1959 geboren te Loon op Zand. Na het behalen van het V.W.O.-b diploma, op het St. Pauluslyceum te Tilburg in 1978, werd in datzelfde jaar gestart met de studie milieuhygiëne/waterzuivering aan de Landbouwhogeschool te Wageningen. Het vakkenpakket in de doctoraalfase bestond uit microbiologie, fysische en kolloïdchemie en waterzuivering. De praktijktijd werd verricht bij het waterschap Regge en Dinkel te Almelo en de Eidgenössische Anstalt für Wasserversorgung und Abwasserreinigung (EAWAG) te Dübendorf, Zwitserland. Na afronding van de studie, in maart 1985, startte de auteur met een driejarig promotie onderzoek bij de vakgroepen microbiologie en fysische en kolloïdchemie van de Landbouwuniversiteit, hetgeen geresulteerd heeft in dit proefschrift. Momenteel is de auteur werkzaam als universitair docent bio-(proces)technologie/milieutechnologie bij de vakgroep bioprocestechnologie van de Technische Universiteit Delft.