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PERMANENT ATTACHMENT OF MYXOBACTERIA MYXOCOCCUS XANTHUS

The permanent attachment of Myxococcus xanthus V-25 and M. xanthus 422 to the hydrophobic and hydrophilic surface was studied. The strains attached mostly to the hydrophobic surface. Protease and chloramphenicol did not promote the cells to detach from both surfaces, but sodium periodate did. This suggests that exopolysaccharide may play a role in their permanent attachment. The presence of Zn, Pb, Cd and Cr induced the changes in the permanent attachment of the strains to both types of the surfaces. It appeared to be reflected in the changes to the charge and hydrophobic characteristics of the cell surfaces measured by hydrophobic and electrostatic interaction chromatography. There was a decline in attachment for death phase cells compared exponential and stationary phase cells but there were no major changes in the cell surface characteristics with growth phase. This suggests that cell physiological activity may contribute to irreversible adhesion for myxobacteria, however, ClCCP, a metabolic inhibitor, did not affect the permanent attachment of the cells. The attachment of both strains declined with increasing pH. Possible mechanisms for the permanent attachment are discussed in the light of these results.

K e y w o r d s: myxobacteria, attachment, hydrophobic and hydrophilic surface

Myxobacteria are gliding bacteria which have complex life cycles involving extensive communication and co-operation between cells e.g. in fructuation [1]. The gliding habit of myxobacteria makes contact with solid surfaces crucial [2], but although the mechanisms for their gliding motility have been much studied, little is known about the other surface contact interactions e.g. the permanent attachment.

Gliding motility involves temporary attachment to a solid surface. *Myxococcus xanthus* Beebe, 1941 shows two distinct forms of gliding motility. The first is movement that involves co-operation between cells and cell-cell proximity termed S-motility. S-motility appears based on the extension pili and their adhesion to a matrix of fibrils, composed of proteins and carbohydrates, followed by the retraction of the pili. The second type of movement, A-motility, allows individual cell movement and is driven by the extrusion of slime. A-motility under some conditions may contribute to group motility. S-motility appears to dominate the movement of *M. xanthus* on soft surfaces while A-motility is more important on hard surfaces

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such as hard agar. The complex developmental life-cycle of myxobacteria requires cell-cell signaling, motility and contact with solid surfaces. In low nutrient conditions vegetative cells aggregate and differentiate into fruiting bodies. S-motility contributes substantially to this process [2, 3]. Other gliding bacteria, such as *Flexibacter sp.*, are capable of both the temporary adhesion, allowing lateral movement, to substrata that is prerequisite of gliding motility and permanent (irreversible) attachment. Permanent attachment is characterized by bacterial cells being firmly attached to one site on the surface even when exposed to the considerable sheer forces. There are two phases in permanent attachment of bacterial cells, reversible and irreversible adhesion. The former is controlled by long-range forces e.g. London-van der Waals forces, while the irreversible adhesion is driven by short-range interactions e.g. hydrophobic and charge interactions between the solid and cell surfaces. The extent of permanent attachment to the surfaces is determined by a number of factors including the characteristics of the bacterial cell surface, the nature of the solid surface and liquid phase [4].

The aim of this study was to investigate the permanent (irreversible) attachment of two *M. xanthus* strains to the hydrophobic and relatively hydrophilic polystyrene surface.

Materials and Methods

Myxococcus xanthus V-25, isolated from Ukrainian soil, and *M. xanthus 422* (provided by Professor J. Ma Arias-Penalver, Granada University, Spain) were used in this study.

CT broth (100 ml) containing 2 % (w/v) casitone (Difco) and 0.2% MqSO, \Box $H_{a}O$ in 0.01M potassium phosphate buffer (pH 7.6) was inoculated with 1 ml M. xanthus from CT starter cultures. The cultures were grown under 30 °C before harvesting (11,000 av. q, 4 °C) and suspending in 10 ml 0.2 mM maleate buffer (pH 7). The cells were washed once and resuspended in maleate buffer (pH 7) to cell density of 1-2 x 10⁹ cells ml⁻¹. Five ml of each cell suspension were transferred to duplicate 5 cm polystyrene petri dishes (PD) (Sterilin), a hydrophobic surface, and duplicate tissue culture treated polystyrene dishes (TCD) (Costar), relatively hydrophilic surface. The attachment substrata were incubated for 2 h at 30 °C and then washed gently three times with 0.2 mM maleate buffer (pH 7) to remove loosely attached bacteria. The attached bacteria were then fixed and stained with crystal violet. Bacterial attachment was estimated by measuring the $\mathrm{A}_{_{540}}$ of the stained attached bacteria. Four readings of randomly selected areas were taken from each of the duplicate dishes. The results were expressed as $\rm A_{540}$ (x 10³) of the attached cells with the 95 % confidence limits of the mean (n=8).

The adhesion assays determined the effect of growth phase on attachment, the effect of selected heavy metals on adhesion, the effect of the inhibitor carbonil cyanide m-chlorophenyl-hydrazone (ClCCP), the effect of pH on adhesion.

To determine the detachment assay the substrata with the attached cells inoculated with 5-ml volumes of one of the following: (i) 0.2 mM maleate buffer (pH 7) (control); (ii) 5 mg chloramphenicol ml⁻¹ (Sigma) in maleate buffer; (iii) 1 unit per 5 ml bacterial protease (Sigma) in maleate buffer; (iv) 1% (w/v) sodium periodate (Sigma). The duplicate plates were incubated at 30 °C for further 2 hrs before washing, staining and determining the A_{540} of attached cells. The results were expressed as the percentage of $A_{540}(x \ 10^3)$ detached by the treatment in comparison with the detachment control. If the 95% confidence limits of the mean (n=8) for treatments and control overlapped then no additional detachment was considered to have occurred.

Cell surface characteristics were investigated by adapting the method previously described [16]. The strains were grown in CT medium at 30 $^{\circ}$ C to



exponential, stationary, or death phase. The cells were harvested, washed once and resuspended in maleate buffer (pH 7) or buffer containing 1.0 mM ClCCP. The strains were also grown for 48 h in CT medium containing 0 mM (control) or 0.1 mM Cr(NO₃)₃· 9H₂O, ZnSO₄· 7H₂O, CdCl₂· H₂O or Pb(NO₃)₂. The cells were harvested by centrifugation, washed once and resuspended in maleate buffer (pH 7) to optical density of $1 - 2 \ge 10^9$ cells ml⁻¹.

The results were expressed as the percentage of A_{540} retained in the column: the larger percentage, the more hydrophobic, anionic, or cationic the cells. Discrepancy of $< \pm 5$ % between duplicate columns was considered acceptable. All experiments were repeated at least once.

Results and discussion

Attachment of both myxobacterial strains was far greater to the hydrophobic surface than the relatively hydrophilic surface in all the growth phases (Table 1). There was no significant difference in permanent attachment between the late exponential phase cells and the stationary phase cells for both strains. There was, however, a significant reduction in the attachment for death phase cells (Table 1). The presence of ClCCP did not inhibit attachment in any growth phase for either strain, in fact there was a slight rise in the attachment induced by ClCCP for death phase cells. *M. xanthus 422* showed higher attachment to the relatively hydrophilic surface (TCD) than *M. xanthus V-25*, but the extent of the attachment to the hydrophobic surface was similar for both strains (Table 1).

Table 1

			A_{540} of attached cells (x 10 ³)					
Strain M. xanthus	Surface	Condition	Exponential Stationary		Death			
			phase cells	phase cells	phase cells			
V-25	PD	Control	27.00 ± 4.00^{a}	30.00 ± 3.00	1.50 ± 0.02			
		+ClCCP ^b	36.00 ± 5.00	31.05 ± 4.00	1.72 ± 0.03			
	TCD	Control	3.00 ± 0.20	3.17 ± 1.00	0.35 ± 0.06			
		+ClCCP	3.00 ± 0.30	3.20 ± 1.00	0.71 ± 0.01			
422	PD	Control	32.00 ± 6.00	35.55 ± 5.00	2.46 ± 0.03			
		+ClCCP	43.00 ± 7.00	36.17 ± 3.00	2.82 ± 0.04			
	TCD	Control	8.00 ± 0.80	8.50 ± 0.80	0.68 ± 0.06			
		+ClCCP	8.90±0.90	8.57 ± 0.60	1.50 ± 0.06			

The effect of growth phase and CICCP on the attachment of *M. xanthus* strains to the hydrophobic surface (PD) and relatively hydrophilic surface (TCD)

^a 95 % confidence limits of mean (n=8)

^b 1 mM carbonil cyanide m-chlorophenyl-hydrazone

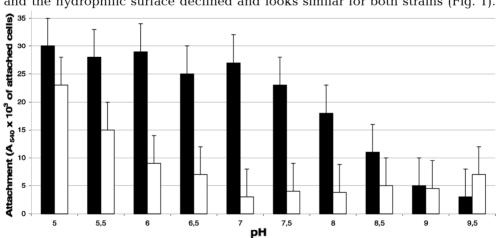
The effect of the heavy metals on the permanent attachment of the two strains was broadly similar, but varied with the solid surface (Table 2). The presence of each metal, in general, significantly increased the attachment of the strains to the relatively hydrophilic surface. There was no rise in attachment to this surface with increasing metal concentration (0,1 and 5,0 mM). The attachment to the hydrophobic surface, however, increased significantly in the presence of 0,1 mM concentrations of all three metals but showed the marked decline at 5,0 mM concentrations of the metals (Table 2).

Table 2

The effect of selected heavy metals on the attachment of <i>M. xanthus</i> strains
to the relatively hydrophilic surface (TCD) and hydrophobic surface (PD)

Strain	Heavy	A ₅₄₀ of attached cells (x 10^3)						
M. xanthus	metal	PD			TCD			
		0,0 mM	0,1 mM	5,0 mM	0,0 mM	0,1 mM	5,0 mM	
	Cr	27.0 ±0.5 ^a	33.0 ± 1.0	16.0 ± 5.0	3.0 ± 0.2	8.0 ± 2.0	7.0 ± 2.0	
	Zn	27.0 ± 0.5	30.0 ± 1.0	15.0 ± 3.0	3.0 ± 0.2	4.0 ± 0.5	4.0 ± 0.1	
	Cd	27.0 ± 0.5	31.0 ± 1.0	15.0 ± 4.0	3.0 ± 0.2	6.0 ± 1.0	5.0 ± 0.5	
V-25	Pb	27.0 ± 0.5	32.0 ± 1.0	16.0 ± 5.0	3.0 ± 0.2	4.0 ± 0.5	4.0 ± 0.1	
	Cr	32.0 ± 1.0	39.0 ± 3.0	18.0 ± 3.0	8.0 ± 0.8	15.0 ± 4.0	15.0±1.0	
	Zn	32.0 ± 1.0	38.0 ± 3.0	15.0 ± 3.0	8.0 ± 0.8	9.0 ± 2.0	10.0 ± 0.5	
	Cd	32.0 ± 1.0	37.0 ± 2.0	15.0 ± 3.0	8.0 ± 0.8	10.0 ± 3.0	12.0±1.0	
422	Pb	32.0 ± 1.0	38.0 ± 2.0	16.0 ± 4.0	8.0 ± 0.8	11.0 ± 0.5	12.0 ± 1.0	

^a 95 % confidence limits of mean (n=8)



As pH increased so the levels of the permanent attachment to the hydrophobic and the hydrophilic surface declined and looks similar for both strains (Fig. 1). 35 $_{-1}$

Fig. 1. Effect of pH on the permanent attachment of *M. xanthus* V-25 to the hydrophobic (PD) surface (\blacksquare) and relatively hydrophilic (TCD) surface (\Box). The bars represent the 95% confidence limits of the mean (n=8)

The attached cells of *M. xanthus* V-25 and 422 did not desorb in the presence of either chloramphenicol, the inhibitor of protein synthesis, or protease. The presence of sodium periodate, which denatures exopolysaccharide, resulted in 30% and 10% detachment of *M. xanthus V-25* from PD and TCD respectively. In the case of *M. xanthus 422* a 40% and 20% detachment occurred from PD and TCD respectively after treatment with sodium periodate.

Cell surface hydrophobicity (measured by HIC) of the two myxobacterial strains was similar and remained relatively constant with growth phase (Table 3). There was more variation in the electrostatic interactions (measured by EIC) for the two organisms. Both strains showed greater binding to the EIC (A+C) exchange resin, which binds both negative and positive groups, than to EIC (A), which binds negative groups, in all three growth phases. This suggests that the cells of both strains tended to be electropositive. In general, the presence of CICCP, the metabolic inhibitor, had little effect on the cell surface characteristics of either strains (Table 3).

Table 3

The effect of growth phase on the cell surface characteristics of <i>M. xanthus</i>
strains measured by electrostatic and hydrophobic interaction

chromatography

Strain	Column ^b	% of cells retained in the columns ^a							
M. xanthus	Column	Exponential		Stationare		Death			
		Growth phase		Growth phase		Growth phase			
		Control	ClCCP ^c	Control	ClCCP ^c	Control	CICCP ^c		
V-25	HIC	84 ± 4^{d}	90 ± 5	88 ± 5	90 ± 6	96 ± 8	97 ± 6		
	EIC(A+C)	73 ± 4	69 ± 3	74 ± 5	71 ± 3	95 ± 8	73 ± 7		
	EIC(A)	53 ± 2	48 ± 3	48 ± 2	46 ± 2	51 ± 4	70 ± 7		
	HIC	80 ± 4	85 ± 5	90 ± 6	91 ± 5	92 ± 6	95 ± 6		
422	EIC(A+C)	60 ± 3	50 ± 2	85 ± 6	80 ± 7	91 ± 8	90 ± 6		
	EIC(A)	50 ± 2	45 ± 2	55 ± 2	50 ± 1	65 ± 3	60 ± 4		

 $^{\rm a}$ Calculated as the percentage of ${\rm A}_{\rm 540}$ retained in the column.

^b HIC, hydrophobic interaction chromatography column; EIC(A+C), mixed anion and cation interaction column; EIC(A), anion interaction column.

^c 1 mM carbonil cyanide m-chlorophenyl-hydrazone

^d SEM (n=3)

The presence of Cr, Zn, Cd and Pb increased both the hydrophobic and charge characteristics of the M. xanthus V-25 and M. xanthus 422 cell surfaces (Table 4). The most substantial increasing was found for EIC (A) suggesting the increase in the positive charge of the cells in the presence of the metals (Table 4).

Table 4

Strain	Column ^b	% of cells retained in the columns ^a					
	Column	Control	Cr	Zn	Cd	Pb	
	HIC	90 ± 1^{c}	97 ±2	99 ± 1	98 ± 2	99 ± 1	
V-25	EIC(A+C)	70 ± 3	88 ± 2	87 ± 3	90 ± 4	79 ± 4	
	EIC(A)	50 ± 3	90 ± 2	90 ± 4	100 ± 1	83 ± 2	
	HIC	90 ± 1	99 ± 1	97 ± 4	96 ± 2	97 ± 3	
422	EIC(A+C)	70 ± 2	81 ± 3	88 ± 3	86 ± 2	85 ± 3	
	EIC(A)	55 ± 4	90 ± 4	92 ± 4	87 ± 1	89 ± 3	

The effect of heavy metal cations on the cell surface characteristics of *M.xanthus* strains measured by electrostatic and hydrophobic interaction chromatography

 $^{\rm a}$ Calculated as the percentage of $A_{_{\rm 540}}$ retained in the column.

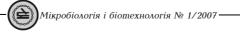
^b HIC, hydrophobic interaction chromatography column; EIC(A+C), mixed anion and cation interation column; EIC(A), anion interaction column.

 $^{\circ}$ SEM (n=2)

This research demonstrates that *M. xanthus* strains not only exhibit the temporary adhesion of gliding motility but are also capable of permanent attachment to solid substrata. Our results suggest that the mechanism of permanent attachment of these organisms may involve complex interactions between several different cell characteristics.

M. xanthus 422 and *M. xanthus V-25* both showed far greater permanent adhesion to the hydrophobic surface than to the relatively hydrophilic surface (Table 1 and Table 2, Fig. 1). Hydrophobic interactions are believed to be key determinants of bacterial attachment [1], that is well known that cell surface charge is only important at cell low surface hydrophobicity. The myxobacterial strains studied both had high surface hydrophobicity and, we are sure cell hydrophobicity might be considered largely responsible for their permanent attachment. This is supported by the greater adhesion to the hydrophobic surface, and by the fact that cells became more hydrophobic, as well as more highly charged (Table 4), after accumulating all the heavy metals studied, when they also tended to show increased adhesion (Table 2).

Hydrophobicity was not, however, the only determinant of permanent attachment for the two *M. xanthus* strains. This was indicated by the findings that death phase cells attached least (Table 1), despite these cells have similar hydrophobicity to stationary and exponential phase cells (Table 3). Moreover, exposure to 5 mM metal solutions resulted in reduced attachment to the hydrophobic but not the relatively hydrophilic surface (Table 2), even though the cell surface hydrophobicity increased after exposure to the metals (Table 3). In their classic study Marshall et al [4] proposed activly related adhesion process specifically through the production of exopolymer. A role for bacterial activity and exopolymer production in permanent attachment has subsequently been supported by a number of studies [1, 5]. The reduced attachment of the



myxobacterial strains we observed in death phase may have been due to some activity driven component in their permanent attachment. The metabolic inhibitor, ClCCP, however, did not reduce their attachment (Table 1). The life-cycle of myxobacteria requires sophisticated co-operation and aggregation of cells largely controlled by cell-cell signaling [3]. Communication and co-operation between cells has been suggested to be exceptionally important in the permanent attachment of bacteria [6]. The apparent contradiction between the reduced attachment for death phase cells but no similar reduction for ClCCP-treated cells, may be due to different effects on the functioning of inter-cell communication and co-operation mechanisms. The role of cell-cell communication and co-operation in permanent attachment of these micro-organisms certainly warrants further study.

The results of our investigation suggest that exopolymer contributes to the irreversible attachment of *M. xanthus* and it is probable that a key effect of metals on attachment was through an effect on exopolymer. Neither chloramphenicol nor protease caused the detachment of the myxobacteria permanently attached to the solid surfaces, but sodium periodate, which denatures exopolysaccharide, did. Attachment of the strains to the hydrophobic surface tended to decline with increasing pH (Fig. 1), as was found for Flexibacter sp, another type of gliding bacterium [18]. The effects of pH are, of course, multifaceted, but it is noteworthy that changes in pH can cause the alterations in the viscosity of exopolymer. Similarly, binding of metal cations to exopolymer might increase its viscosity. Cadmium bound to the exopolymer of Citrobacter sp. changed the morphology of the exopolymer from diffuse and amorphous to electron opaque and structured [7]. Viscosity has been suggested as one factor that determines whether exopolymer acts as a temporary adhesive in gliding motility or as a permanent adhesive [8]. Binding of metals to bacterial cell walls also substantially changes the charge and hydrophobic characteristics of the cell surface [5], which in turn may influence attachment. In the case of myxobacteria we found that there was increasing in both hydrophobicity and charge interactions, particularly the positive charge interactions of the cells (Table 4). Cadmium and zinc have previously been found to cause similar increases in the charge and hydrophobic characteristics of the cell surface of Pseudomonas fluorescens H2 [5]. In addition, the presence of the metals had a considerable impact on the physiological activity of the strains, clearly demonstrated by the reduction in respiration rate. Variations in the physiological status of bacteria are known to induce changes in the cell surface characteristics [5]. Metal induced changes in the cell surface characteristics of the *Myxococcus* strains are likely to have impacts on the cell-surface interactions permanent adhesion influencing the levels of attachment as observed in this study.

It is clear from our research that myxobacteria are capable permanent attachment to solid surfaces and that a number of different cellular characteristics contribute to this process. The life-style and gliding motility of these bacteria makes the contact with the surfaces essential. Whether the ability to irreversibly attach plays a role for *M. xanthus* in soil environments is unclear, but is worthy of further study.

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ДОЛГОСРОЧНОЕ ПРИКРЕПЛЕНИЕ МИКСОБАКТЕРИЙ *МУХОСОССUS XANTHUS*

Реферат

Изучено долгосрочное прикрепление бактерий *Мухососсиs xanthus* V-25 и *M. xanthus* 422 к гидрофобным и гидрофильным поверхностям. Показано, что миксобактерии лучше прикреплялись к гидрофобным поверхностям. Периодат натрия способствовал откреплению от обоих типов поверхностей, в отличие от протеазы и хлорамфеникола. Присутствие Zn, Pb, Cd и Cr приводило к изменениям в прикреплении штаммов к обоим типам поверхностей. Наблюдалось снижение прикрепления в фазе гибели клеток, по сравнению с экспоненциальной и стационарной фазами. Существенных отличий в изученных свойствах клеточной поверхности на различных фазах роста не выявлено. Прикрепление бактерий обоих штаммов снижалось с повышением pH. В свете полученных результатов обсуждены возможные механизмы долгосрочного прикрепления миксобактерий.

Ключевые слова: миксобактерии, прикрепление, гидрофобные и гидрофильные поверхности.



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ДОВГОТРИВАЛЕ ПРИКРІПЛЕННЯ МІКСОБАКТЕРИЙ *МҮХОСОССUS XANTHUS*

Реферат

Вивчено довготривале прикріплення бактерій *Мухососсиs xanthus* V-25 і *M. xanthus* 422 до гідрофобних і гідрофільних поверхонь. Показано, що міксобактерії краще прикріплювалися до гідрофобних поверхонь. Періодат натрію сприяв відкріпленню від обох типів поверхонь, на відміну від протеази та хлорамфеніколу. Присутність Zn, Pb, Cd і Cr призводила до змін в прикріпленні бактерій до обох типів поверхонь. Спостерігалось зниження прикріплення у фазі загибелі клітин, в порівнянні з експоненціальною та стаціонарною фазами. Суттєвих відмін вивчених властивостей поверхні клітин на різних фазах росту не виявлено. Прикріплення бактерій обох питамів знижувалось з підвищенням pH. В світлі одержаних результатів обговорено можливі механізми довготривалої адгезії міксобактерій.

Ключові слова: міксобактерії, прикріплення, гідрофобна і гідрофільна поверхні.