JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 776–779
0021-9193/84/080776-04$02.00/0

Vol. 159, No. 2

Changes in Cell Surface Hydrophobicity of *Myxococcus xanthus* Are Correlated with Sporulation-Related Events in the Developmental Program

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Received 26 March 1984/Accepted 7 May 1984

Cell surface hydrophobicity was measured in the bacterium *Myxococcus xanthus* during vegetative growth, fruiting body formation, and glycerol-induced spore formation by the method of Rosenberg et al. (FEMS Microbiol. Lett. 9:29–33, 1980). A significant decrease in cell surface hydrophobicity was observed 12 to 36 h after fruiting body formation and 60 to 120 min after glycerol-induced sporulation. The hydrophilic shift was correlated with the ability of the cells to sporulate but not with their ability to aggregate. Sucrose gradient purification removed the hydrophilic substance from the fruiting body spores but not from the glycerol-induced spores. The change in cell surface hydrophobicity in *M. xanthus* should be a useful developmental marker.

Myxococcus xanthus is a gram-negative, rod-shaped bacterium which undergoes complex cell-cell interactions (6, 14; D. R. Zusman, Q. Rev. Biol., in press). When *M. xanthus* is grown under starvation conditions on a solid surface at high cell densities, a developmental program is triggered which results in cellular aggregation and sporulation (7; D. R. Zusman, *in* E. Rosenberg, ed., *Development and cell interactions in myxobacteria*, in press). Aggregation involves the movement of cells toward specific sites, where 10^5 to 10^6 cells form raised mounds in which sporulation occurs. The sporulation program controls the conversion of individual rod-shaped cells to round, environmentally resistant resting cells called myxospores. For *M. xanthus*, these mounds of myxospores are termed fruiting bodies. Dworkin and Gibson (3) discovered an additional sporulation mechanism in *M. xanthus*. Adding high concentrations of glycerol (0.5 M), several alcohols, or dimethyl sulfoxide (D. Zusman, reported in reference 1) to exponential-phase cultures causes the cells to become refractile, sonication-resistant, heat-resistant "spores"; removing the glycerol by dilution into fresh medium results in synchronous germination (9) and cell division (15). However, the sporelike cells which result from this induction process differ in many respects from the spores obtained from fruiting bodies (14). In this paper we report changes in the cell surface hydrophobicity of *M. xanthus* during fruiting body formation and glycerol-induced sporulation.

Rosenberg et al. (10) described a simple method for measuring cell surface hydrophobicity based on the adherence of bacteria to hydrocarbons. The assay consists of adding a small amount of a hydrocarbon to a turbid bacterial culture, agitating the suspension for 2 min in a Vortex mixer, and then allowing the suspension to settle for 15 min to permit phase separation. The decrease in absorbance of the aqueous lower phase measured at 400 nm is then used as a measure of the cell surface hydrophobicity. This assay was used for *M. xanthus* vegetative cells, fruiting body spores, and glycerol-induced spores with hexadecane or xylene as the hydrocarbon. Under the conditions used in the assay, cell lysis was not observed. However, vegetative cells showed an approximately 10-fold loss in viability after hexadecane treatment and a 10^6-fold loss after xylene treatment, whereas the spore samples showed only slight losses in viability after exposure to these hydrocarbons (twofold with hexadecane and fivefold with xylene). Vegetative cells showed significant cell surface hydrophobicity (Fig. 1). Approximately 50% of the cells adhered to the nonaqueous phase in the presence of hexadecane, and 80 to 90% of the cells adhered to the nonaqueous phase in the presence of xylene. Rosenberg et al. (10) found no significant adherence of *Escherichia coli* B cells to hexadecane and about 20% adherence to xylene; in contrast, the oil-emulsifying bacterium *Acinetobacter calcoaceticus* RAG-1 showed about 90% adherence to both hexadecane and xylene. Fruiting body spores and glycerol-induced spores from *M. xanthus* had a cell surface hydrophobicity very different from that of vegetative cells (Fig. 1B and C). Less than 10% of these spores adhered to either hexadecane or xylene. Sporulation therefore results in profound changes in the cell surface properties of *M. xanthus*, as measured by the Rosenberg cell surface hydrophobicity assay.

The changes in the cell surface properties during fruiting body formation were studied in more detail (Fig. 2). *M. xanthus* cells were spotted onto clone fruiting agar, and cells were harvested at intervals and assayed for cell surface hydrophobicity and for the presence of heat-resistant spores. When hexadecane was used as the hydrocarbon in the assay, there was a dramatic change in cell surface properties between 12 and 36 h after development began. This time corresponds to the period of cellular aggregation into mounds, but clearly precedes sporulation (the cells were rod shaped and sensitive to sonication and heat). When xylene was used as the hydrocarbon (data not shown), the observed changes in hydrophobicity were even greater but took place about 8 to 12 h later. The changes in cell surface properties during glycerol-induced spore formation occurred between 60 and 120 min after the glycerol was added, when the surface properties of the cells rapidly changed from hydrophobic (60 to 70% adherence to hexadecane, 80% adherence to xylene) to hydrophilic (10% adherence to hexadecane and to xylene) (Fig. 3). The time of this change corresponded to the time at which morphogenesis from long rods to ovoid cells occurred.

Since such major changes in the cell surface properties of *M. xanthus* were observed during development, it was of interest to examine mutants blocked in development (sporu-

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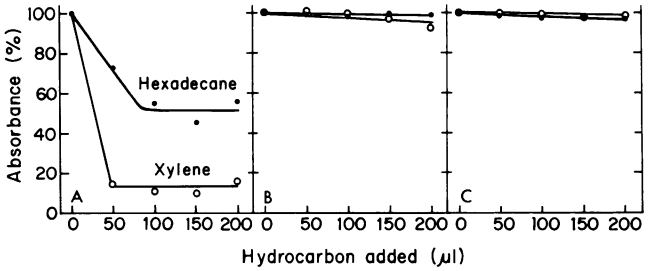

FIG. 1. Adherence of vegetative cells (A), fruiting body spores (B), and glycerol-induced spores (C) of *M. xanthus* to hydrocarbons. *M. xanthus* FB (DZF1) was grown vegetatively to about 2.5×10^8 cells per ml in Casitone-yeast extract broth (2) at 30°C. Development was induced by spotting cells on clone fruiting agar (4) as previously described (8); the spores were harvested after 7 days and dispersed by sonification for 2 min. Glycerol-induced spores (24 h after glycerol addition) were prepared as described by Morrison and Zusman (7). The vegetative cells and spores were harvested, washed twice with PUM buffer (10), vortexed after each wash, and then assayed for cell surface hydrophobicity. Briefly, the cells were suspended in PUM buffer to an OD_{400} of 0.5 to 0.8. Portions (1.2 ml) were placed in 10-mm-diameter test tubes, and 0, 50, 100, 150, or 200 μl of hexadecane (●) or xylene (○) was added to the tubes. The samples were incubated for 5 to 10 min at 30°C and then agitated with a Vortex mixer for 2 min at the highest speed. After 15 min of incubation at room temperature, the lower (aqueous) phase was removed and the OD_{400} was measured. The percent absorbance of the lower phase compared with the initial absorbance indicates the relative adherence of the cells to the hydrocarbon.

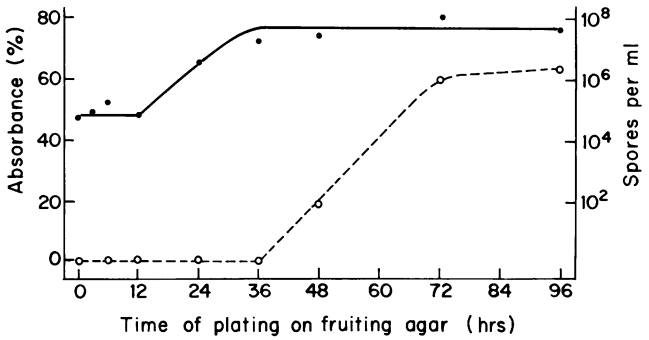

FIG. 2. Changes in cell surface properties of *M. xanthus* during fruiting body formation. *M. xanthus* DZF1 was spotted onto clone fruiting agar as described in the legend to Fig. 1, and at the indicated times samples were removed (8) and assayed for cell surface hydrophobicity with hexadecane and for the presence of heat-resistant spores. The data were first plotted as shown in Fig. 1. From each plot, we drew the best line through the limit values (usually at 100, 150, and 200 μl of hexadecane). The percent absorbance at these limit values is plotted against time (●). The number of viable heat- and sonication-resistant spores at each time point is also shown (○).

lation or aggregation) (Table 1). Aggregation-defective, sporulation-proficient mutants underwent the shift from hydrophobic to hydrophilic cell surface properties, behaving like wild-type cells. However, mutants blocked in sporulation failed to undergo this shift. For example, the sporulation-defective strains DZF1098, DZF1760, and DZF1963, all of which are temperature-sensitive sporulation mutants, showed normal sporulation and hydrophobicity values at 28°C but did not undergo the hydrophilic shift at 34°C. One exception to these findings was the SpoD strain DK429, a synergistic sporulation mutant, which did not sporulate but did undergo a partial hydrophilic shift when grown on fruiting agar. This shift seems to be significant because DK3260, a strain constructed by transducing the SpoD allele of strain DK429 into strain DK1622, underwent an even greater shift during fruiting body formation (hydrophobicity values shifting from 70 to 10%).

The data for the glycerol-induced spores are more complex. Many sporulation-defective (Spo^-) mutants (e.g., SpoA through SpoD) are able to form spores in the presence of glycerol even though they cannot sporulate on fruiting agar; these mutants did, in fact, undergo the hydrophilic shift. However, two of the three temperature-sensitive Spo^- mutants underwent this shift even though they were unable to form spores in the presence of glycerol. Observation of these mutants under the microscope showed that after glycerol addition at 34°C, they underwent a transitory conversion from rod-shaped cells to shortened rods or ovoid cells; at later times the cells were all rod shaped. It therefore appears likely that these two temperature-sensitive Spo^- mutants are blocked after a step which causes a modification in the hydrophobic properties of the cell surface, but before the formation of refractile glycerol-induced spores.

In what way is the surface of *M. xanthus* modified during development? To address this question, we purified fruiting body and glycerol-induced spores on sucrose gradients. To our surprise, this treatment completely eliminated the hydrophilic character of the fruiting body spores but had no effect on the glycerol-induced spores (Fig. 4). These results suggest that some hydrophilic material, possibly slime or capsular material, may have been deposited on the cells during fruiting body formation and that this material was not covalently bound to the spores since it could be removed by sucrose gradient purification; however, the hydrophilic modification(s) of glycerol-induced spores may differ since the hydrophilic material could not be easily removed from these spores.

In reviewing these experiments, we became concerned that cellular debris from cell lysis (developmentally induced lysis [13] and sonication) might be distorting the results of

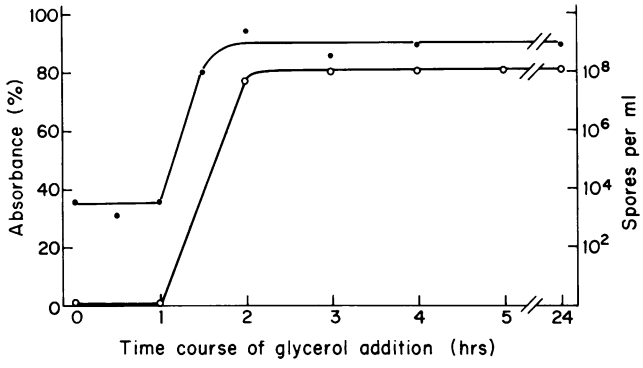

FIG. 3. Changes in cell surface properties of *M. xanthus* during spore induction by glycerol. *M. xanthus* DZF1 cells were grown to about 1.5×10^8 cells per ml in Casitone-yeast extract broth. Glycerol was then added to 0.5 M. Samples were incubated with shaking for 24 h at 20°C. At the indicated times, portions were removed and measured for cell surface hydrophobicity with hexadecane as the hydrocarbon. The data were analyzed as described in the legend to Fig. 2. The percent absorbance values (from the limit values) are plotted against the time after glycerol addition (●). The spore counts obtained with the Petroff-Hauser counter at each time are also shown (○).

TABLE 1. Cell surface properties of *M. xanthus* mutants blocked in sporulation or aggregation[a]

Strain and reference	Growth temp (°C)	Phenotype[b]	Fruiting agar			Glycerol induction medium		
			Hydrophobicity[c] at:		Spores formed	Hydrophobicity at:		Spores formed
			0 h	72 h		0 h	24 h	
Wild-type								
DK1622 (11)	30	Fruiting (A^+ S^+ motile)	50	22	Yes	65	2	Yes
DZF1 (=DK101) (5, 7)	30	Fruiting (A^+ S^- motile)	50	10	Yes	35	1	Yes
	28	Fruiting	55	14	Yes	60	1	Yes
	34	Fruiting	60	30	Yes	60	14	Yes
Sporulation mutants								
DK480 (4)	30	SpoA	44	41	No	35	3	Yes
DK468 (4)	30	SpoB	44	45	No	36	5	Yes
DK731 (4)	30	SpoC	41	48	No	37	5	Yes
DK429 (4)	30	SpoD	43	27	No	38	10	Yes
DZF1098 (7)	34	Tm (Spo^-)	43	43	No	43	15	No
	28	Fruiting	43	10	Yes	43	6	Yes
DZF1760 (7)	34	Tm (Spo^-)	50	58	No	50	38	No
	28	Fruiting	50	10	Yes	50	24	Yes
DZF1963 (7)	34	Tm (Spo^-)	62	50	No	62	17	No
	28	Fruiting	62	8	Yes	62	11	Yes
Aggregation mutants								
DZF1516 (12)	34	AggR1	73	12	Yes	73	1	Yes
	28	Fruiting	73	39	Yes	73	27	Yes
DZF1552 (12)	34	AggR2	61	13	Yes	61	26	Yes
	28	Fruiting	61	3	Yes	61	0	Yes
DZF1287 (12)	34	AggR3	62	23	Yes	62	17	Yes
	28	Fruiting	62	3	Yes	62	0	Yes
DZF1084 (16)	30	Frizzy	50	1	Yes	70	2	Yes
DZF1281 (16)	30	Frizzy	38	8	Yes	70	0	Yes
DZF1961 (16)	30	Frizzy	55	2	Yes	62	2	Yes

[a] Cells were grown at 28, 30, or 34°C and then spotted onto clone fruiting agar to induce fruiting body formation (72 h) or treated with glycerol (0.5 M, 24 h) to induce spores.

[b] Abbreviations: A and S, motility (the two motility systems of *M. xanthus* are described in reference 5); Tm, sporulation defect (mutants form translucent mounds); AggR, temperature-sensitive aggregation; frizzy, abnormal motility and ribbonlike aggregates.

[c] The data were obtained with the Rosenberg assay (10) and plotted as percent absorbance versus hexadecane concentration as shown in Fig. 1. From each plot, we drew the best line through the limit values (usually at 100, 150, and 200 μl of hexadecane). Hydrophobicity values thus represent the fraction of cells lost (percent reduction in OD_{400}) after hydrocarbon extraction.

the Rosenberg hydrophobicity assay by interfering with the normal relationship between the number of spores and the optical densities at 400 nm (OD_{400}). Thus, if most of the absorbance measured at 400 nm were due to cell debris rather than to spores, the lack of change in the OD_{400} after hexadecane treatment would reflect lack of adherence of cell debris to the hydrocarbon rather than lack of adherence of spores to it.

To test this hypothesis, we determined the number of spores directly in a Petroff-Hausser counting chamber. The number of spores counted after hexadecane treatment (6.3×10^8/ml) was, within experimental error, identical to that counted before treatment (6.4×10^8/ml). Therefore, the spores present in the crude spore preparation showed no measurable adherence to hexadecane. This supports our conclusion that the sucrose gradient purification procedure must have removed a hydrophilic surface component present on the fruiting body spores.

The results presented in this paper are interesting in relation to the developmental program of *M. xanthus* because they provide evidence of a timed change in the cell surface properties of this organism during fruiting body formation and spore induction by glycerol. This change appears to be correlated with sporulation-related events in

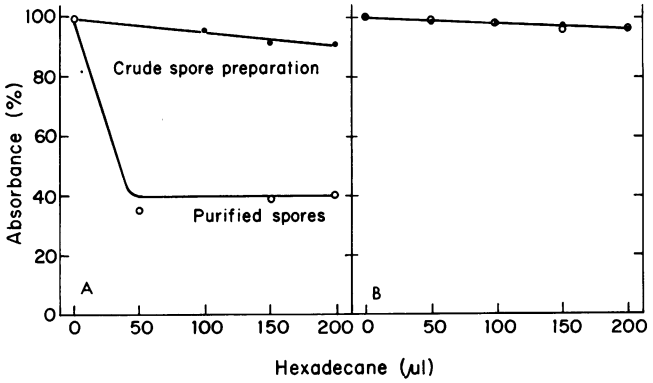

FIG. 4. Effect of sucrose gradient purification of spores on cell surface hydrophobicity. Crude spore preparations (sonicated spores) were purified on a sucrose gradient (a step gradient consisting of 5-ml aliquots of 60, 30, 15, and 7.5% sucrose in TM buffer [7], centrifuged at 2,000 rpm for 6 min in a Sorvall HS4 swinging bucket rotor). The spore fraction was resuspended by sonication and assayed for cell surface hydrophobicity with hexadecane as described in the legend to Fig. 2. (A) Fruiting body spores. Symbols: ●, 7-day-old crude spores; ○, purified spores. (B) Glycerol-induced spores. Symbols: ●, 24-h-old induced spores; ○, purified spores.

the developmental program, because mutants blocked in sporulation are usually also blocked in the cell surface change(s), whereas mutants blocked in aggregation but not sporulation behave like the wild type. We therefore suggest that cell surface hydrophobicity is another useful parameter for classifying development mutants of *M. xanthus*.

The timing of the shift from hydrophobic to hydrophilic cell surface properties is also of interest. During fruiting body formation, the shift occurs at 12 to 36 h after plating on fruiting agar, with hexadecane as the hydrocarbon. The timing corresponds to the formation of large mounds and precedes spore formation by many hours. The observation that the hydrophilic shift precedes sporulation helps explain the finding that the SpoD mutant strain DK429 underwent a change in its surface properties without forming mature spores. It should be noted that the cells adhere to each other at this time (sticky), and extensive agitation is needed to achieve some measure of suspension. However, even the clumps of cells are more hydrophilic than the vegetative cells are. This change in the surface properties of the spores may be important in nature for spore dispersal. Since the hydrophilic surface component can be removed from the spores by purification on a sucrose gradient, it cannot be covalently bound to them. It should therefore be possible to purify and characterize this substance. The change in the hydrophobicity of glycerol-induced spores observed in these experiments may be due to a different surface modification(s), since the hydrophilic surface component cannot be removed on a sucrose gradient. Alternatively, the same substance(s) may be produced but bound more tightly to the cell surface in glycerol-induced spores. It is of interest that *Bacillus* spores, in contrast to spores of *M. xanthus*, appear to be much more hydrophobic than vegetative *Bacillus* cells (E. Rosenberg, personal communication). Clearly, further characterization of the cell envelope of *M. xanthus* during the various phases of its life cycle must be made before the significance of the cell surface changes observed in this study can be determined.

We thank Eugene Rosenberg for explaining his assay to us and Dale Kaiser for providing us with strains. We also thank Brian Blackhart, John Downard, and Kathleen O'Connor for helpful discussions.

This research was supported by Public Health Service grant GM20509 from the National Institutes of Health and by grant PCM 8300626 from the National Science Foundation.

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