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Effects of Exopolysaccharide Production on Liquid Vegetative Growth, Stress Survival and Stationary Phase Recovery in *Myxococcus xanthus*

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Abstract

Exopolysaccharide (EPS) of *Myxococcus xanthus* is a well-regulated cell surface component. In addition to its known functions for social motility and fruiting body formation on solid surfaces, EPS has also been proposed to play a role in multi-cellular clumping in liquid medium, though this phenomenon has not been well studied. In this report, we confirmed that *M. xanthus* clumps formed in liquid were correlated with EPS levels and demonstrated that the EPS encased cell clumps exhibited biofilm-like structures. The clumps protected the cells at physiologically relevant EPS concentrations, while cells lacking EPS exhibited significant reduction in long-term viability and resistance to stressful conditions. However, excess EPS production was counterproductive to vegetative growth and viable cell recovery declined in extended late stationary phase as cells became trapped in the matrix of clumps. Therefore, optimal EPS production by *M. xanthus* is important for normal physiological functions in liquid.

Keywords

Myxococcus xanthus; exopolysaccharide; vegetative growth; stress survival; stationary phase recovery

Introduction

Myxococcus is a group of Gram-negative soil bacteria with complex life styles (Reichenbach, 1993). This study focuses on exopolysaccharide (EPS), a key component of *Myxococcus* extracellular matrix (ECM) (Behmlander and Dworkin, 1994; Dworkin, 1993), which is distributed over the entire cell surface of wild-type cells (Merroun *et al.*, 2003). The current understanding of *Myxococcus* EPS mainly comes from the studies of *M. xanthus* behaviors on solid surfaces, where EPS plays important roles in fruiting body formation (Lux *et al.*, 2004; Shimkets, 1986), social (S−) motility (Li *et al.*, 2003; Lu *et al.*, 2005), cell-cell cohesion (Arnold and Shimkets, 1988), cell-substratum adhesion, and protection from adverse environmental factors (Dworkin, 1993; Merroun *et al.*, 1998). It is also known that the production of EPS in *M. xanthus* is well regulated by different genetic loci (Yang, 2008),

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such as the *dif* chemotaxis-like operon (Yang *et al.*, 1998; Yang *et al.*, 2000); *pilA*, a gene encoding the pilus structural protein (Black *et al.*, 2006); *eps* and *eas* regions, encoding proteins for polysaccharide biosynthesis (Lu *et al.*, 2005); and *stkA* and *sglK,* encoding DnaK homologues (Dana and Shimkets, 1993; Yang *et al.*, 1998).

The functions of EPS in *Myxococcus* liquid cultures are much less studied. Due to EPS production, many strains of *Myxococcus*, especially newly isolated ones from soil (Wall *et al.*, 1999), fail to grow as dispersed cells in liquid culture, and form thin films or clumps (Schurmann, 1967), which often hinders laboratory examination or genetic manipulation. This phenomenon has proposed to be associated with the production of EPS, as wild-type *M. xanthus* cells stick together to form clumps in liquid medium (Kim *et al.*, 1999), while cells defective in EPS production, such as SW504 (Δ*difA*) and SW810 (Δ*epsA*), can stay dispersed in liquids (Lu *et al.*, 2005; Yang *et al.*, 1998).

In this study, we are interested in that if different level of EPS production may affect *M. xanthus* physiology in liquid, since some previous studies in other bacteria indicate the similarities between bacterial cells within typical biofilms and cells within the aggregates in liquid (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004), while profound difference between planktonic and biofilm cells (Bhinu, 2005). The utilization of confocal laser scanning microscopy (CLSM) (Neu *et al.*, 2010) and conjugated lectins (Flemming and Wingender, 2010) allows for direct visualization of EPS and cells *in situ* (Lux *et al.*, 2004) and avoids the collapse of EPS which frequently occurs during the fixation and dehydration procedures for electron microscopic observation (Merroun *et al.*, 2003). Combined with other microbiological and physical chemical assays, we investigated the effects of EPS production on liquid vegetative growth, stress survival and stationary phase recovery of *M. xanthus* by analyzing various mutants that produce negligible or excess EPS.

Materials and methods

Bacterial strains, media and growth conditions

To test the viability phenotypes of *M. xanthus* cells, different strains (listed in Table 1) were grown at 32 °C in casitone-yeast extract (CYE) medium (Campos *et al.*, 1978) at 300 rpm for 1 d. The cells were harvested and the cell clumps were dissociated by vortexing in the presence of 3 mm glass beads, resuspended to 1×10^9 cells/ml with fresh CYE. Subsequent culturing was performed at 32 °C on a rotary shaker at 300 rpm. At various time points, cells and clumps were taken, dispersed by voretxing with glass beads and tested for viability by counting colony-forming unit (CFU) as previously described (Nelson and Killeen, 1986). Since the EPS production in *M. xanthus* is stationary-phase dependent (Kim *et al.*, 1999), in order to investigate the long-term viability of these strains, a high cell concentration (1×10^9 cells/ml) was used for inoculation, which allowed the different strains to enter stationary phase rapidly and eliminated possible differences in the initial growth stages.

For the mixed culture experiments, about 8.0×10^7 SW505 (*difA*::Tn5, Km^r) cells were inoculated into 5 ml CYE together with 4.0×10^8 DK1622 (Wt) cells, and incubated at 32 °C on a rotary shaker at 300 rpm for 12 d. As a negative control, 4.8×10^8 SW505 cells were inoculated into 5ml CYE and 4.8×10^8 DK1622 cells were inoculated into 5 ml CYE as a positive control. At various time points, cells of SW505/DK1622 and SW505 were assayed for viable SW505 cells by CFU counting on CYE agar containing 100 μg/ml kanamycin, and cells of DK1622 were assayed for viable DK1622 on CYE agar.

For the re-growth test, the cell clumps of DK1622 (Wt) or DK3088 (*stk*) were directly removed from 4 d cultures and gently washed twice in fresh CYE. Cell clumps (about 15 mg of dry biomass) were either directly inoculated into 50 ml fresh CYE medium or dispersed

by vortexing with glass beads prior to inoculation. These cultures were incubated at 32 °C at 50 rpm and cell growth was monitored by determining the dry biomass. The biomass was collected by 13,000 ×g centrifugation and dried at 80 °C for 24 h.

Trypan blue binding assay

EPS production of different strains in CYE liquid was measured using a trypan blue binding assay (Black and Yang, 2004). The relative amounts of EPS were calculated relative to trypan blue bound to the wild-type DK1622 cells.

Agglutination assay

The cohesion of *M. xanthus* cells was measured with an agglutination assay described by Shimkets (Shimkets, 1986; Shimkets, 1986). The percentage of agglutination was calculated as the ratio of OD_{600nm} at different time point versus initial absorbance at 600 nm.

Measurement of viscosity and rheology

Cells of different strains were harvested from 1 d liquid CYE cultures. EPS were isolated and purified from 5×10^{10} cells according to the protocol previously described (Chang and Dworkin, 1994; Li *et al.*, 2003), and suspended in 2 ml 0.3 M AcOH/0.05 M AcONa/4 mM $CaCl_2$ buffer to generate a homogeneous gel suspension. The efflux times of the EPS suspensions and solvent were measured using an Ubbelohde capillary viscometer (DI10070-75) in a constant-temperature water bath at 32 ± 0.5 °C. The specific viscosity (η_{sp}) was calculated as:

$$\eta_{sp}=(\eta-\eta_0)/\eta_0 \quad [1]$$

where η is the dynamic viscosity of an EPS suspension and η_0 is the dynamic viscosity of buffer.

The solvent used for rheological experiments was MMC buffer (10 mM MOPS, 8 mM $MgSO_4$, 4 mM $CaCl_2$). The lyophilizated EPS isolated from wild-type DK1622 cells were crushed in a mortar, weighted and suspended in the buffer. Then, WT-EPS suspensions with different concentrations were incubated at 32 °C for 48 hr. The measurement of apparent viscosity of EPS suspensions was performed on a LDV-III Ultra rheometer (Brookfield, US) equipped with a LV-1 spindle and an UL-adapter. The influence of shear rate on rheological curves of EPS suspensions was determined at 32 ± 0.1 °C.

Sample preparation and staining method

At different time points, cell clumps were directly isolated from liquid cultures of EPS^+ strains while the cell pellets were collected from EPS^- strains following 13,000 ×g centrifugation for 5 min. The cell-membrane-permeant nucleic acid binding dyes, SYTO 9 or SYTO 82 (both at 5 μM, Molecular Probes, USA), were used to differentiate cells from debris and matrix. 5 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Molecular Probes), a red fluorescent indicator dye of respiratory activity, was used to reveal metabolically active cells. Carbohydrates present in the EPS portion of the cell clumps or pellets were stained with 5 μg/ml of Alexa 633-conjugated derivatives of wheat germ agglutinin lectin (WGA, Molecular Probes) as previously described (Lux *et al.*, 2004). Before the examination under CLSM, the specimens were incubated with dyes in MOPS buffer (10 mM MOPS, 8 mM $MgSO_4$, pH 7.6) for 30 min in the dark. The 12 d clumps formed by EPS^+ cells were incubated with 5 mM CTT in CYE medium for 6 h, then CYE medium was removed by aspiration, replenished with MOPS buffer containing SYTO 9 and Alexa 633-WGA, and incubated for 30 min in the dark.

Confocal laser scanning microscopy (CLSM)

CLSM was employed to visualize the *M. xanthus* clumps and pellets with various dye combinations using a PASCAL5 confocal laser scanning microscope (Zeiss, Germany). Excitation at 488 nm in combination with a 505–530 nm band-pass emission filter were used for Gfp and SYTO 9 imaging, respectively. CTC was visualized using 488 nm excitation and a 560–615 nm band-pass emission filter. SYTO 82 signals were visualized using 543 nm excitation with a helium-neon laser and a 560–615 nm band-pass emission filter. Excitation at 633 nm and a 650 nm long-pass emission filter were used to reveal Alexa 633-WGA.

Exposure to UV irradiation

All planktonic and clumped *M. xanthus* cells were harvested from 1 d CYE liquid cultures. The clumps in broth were removed with 500 ×g centrifugation for 10 min, and DK1622 (Wt) planktonic cells were collected by 13,000 ×g centrifugation of supernatant for 5 min. After washing two times with MMC buffer, about 5×10^8 cells or clumps containing equal amount of cells were suspended in 1ml MOPS buffer. The suspensions were transferred into 6-well culture plates and irradiated in a microprocessor controlled UV crosslinker (XL-1000, Spectroline, US) to achieve the inactivation effects as previously described (Sudo and Dworkin, 1969). Viable cell counts were taken before and immediately after UV exposure with the CFU assay described above, and SW505 (*difA*::Tn*5*, Km^r) viable cells were enumerated on CYE agar containing 100 μg/ml kanamycin. All dose-survival experiments with planktonic and clumped cells were done with five different dosages controlled by energy level of crosslinker, and two replicate irradiated suspensions. The UV dosage required for 99.9% inactivation of cells was determined using a method previously described (Chang *et al.*, 1985).

Sodium dodecyl sulphate (SDS) treatment

The preparation of cells and clumps for SDS treatment was identical to that used for UV irradiation described above. After washing, the cells or clumps (about 5×10^8 cells) were suspended in 1 ml MMC buffer containing 0.01% SDS as previously described (Elias and Murillo, 1991), while mock treatment was done with MMC buffer. The suspensions were incubated at 32 °C for 30 min, and the cells or clumps were washed 2 times with MMC buffer to remove residual SDS. The viable cells were numerated with the CFU assay described above, and survival rate of each strain was calculated as the ratio of viable cells numbers in SDS treated samples versus that in mock treated samples. Two replicate experiments were performed.

Results

EPS production influences viability of *M. xanthus* cells during liquid vegetative growth

As a mixture of polysaccharides, the absolute amount of EPS in stationary broth of *M. xanthus* wild-type strain DK1622 was about 127 ± 9 μg of carbohydrate/mg of protein, which was determined as the amount of anthrone-reactive materials (Kim *et al.*, 1999). A trypan blue binding assay was further developed to quantitatively measure the relative amount of EPS of different *M. xanthus* strains (Black and Yang, 2004). Using this assay, EPS production of four *M. xanthus* mutants in liquid medium was determined (Table 1). Strains SW504 (Δ*difA*) and SW810 (Δ*epsA*) are defective in EPS production due to mutations in EPS regulatory and biogenesis genes, respectively (Lu *et al.*, 2005; Yang *et al.*, 2000); DK10410 (Δ*pilA*) produces about 20% of the amount of EPS as DK1622, because PilA positively functions upstream of Dif proteins in regulating EPS production (Black *et*

al., 2006); and mutation of the *stk* locus results in cells with a higher-than-normal level of EPS (Dana and Shimkets, 1993), such as in the strain DK3088.

In liquid cultures (Fig. 1), cells producing negligible or much reduced EPS (SW504, SW810 and DK10410), which grew dispersed without forming aggregates, were not viable after 6 d. In strains producing sufficient EPS (DK1622 and DK3088), cell clumps were observed and viable cells were still detected after 12d. The EPS-overproducing strain DK3088 (*stk*) displayed enhanced survival abilities compared to wild-type strain DK1622. Microscopic examination revealed that the DK3088 cells had formed a significantly greater number of large clumps versus DK1622 cells. These results show a correlation between EPS production with both clumping and long-term viability of *M. xanthus* cells in liquid culture.

Clump forming in *M. xanthus* broth is a consequence of EPS production

The cohesion of different cells in liquid was examined and shown in Fig. 2A. DK1622 (Wt) and DK3088 (*stk*) showed rapid agglutination, while SW504 (Δ*difA*) and SW810 (Δ*epsA*) were defective in cellular cohesion, which is consistent with their EPS production (Table 1) and cell clumping (Fig. 1) phenotypes. However, about 30% agglutination (Fig. 2A) was detected in DK10410 (Δ*pilA*), though stable clumps were not observed in this strain under our shaken culture condition (Fig. 1).

Next, the rheological properties of EPS from different *M. xanthus* strains were further investigated. Because of their large molecular mass, EPS normally yield highly viscous aqueous solutions or suspensions with complicated molecular interactions, which are important chemical and physical features for its function in cell cohesion and matrix formation (Sutherland, 2001). EPS isolated from equal cell numbers of different strains were suspended in buffer to determine their specific viscosity (η_{sp}, equation [1]). As shown in Fig. 2B, DK3088-EPS showed the highest η_{sp}, while suspensions of EPS from DK10410 (Δ*pilA*), SW504 (Δ*difA*) and SW810 (Δ*epsA*) exhibited very low viscosities ($\eta_{sp} < 0.05$). Although DK10410 (Δ*pilA*) cells still produce EPS and exhibit agglutination to a certain degree (Fig. 2A), the non-clumping phenotype in shaken liquid might be due to the low viscosity of its EPS. After examining the apparent viscosity of a DK1622-EPS (WT-EPS) suspension, a clear concentration-dependent viscosity against shear force was observed (Fig. 2C). At low concentrations (≤ 0.5 g/L), the viscosity was greatly decreased and the EPS suspensions exhibited a non-Newtonian behavior of shear thinning fluids, which was consistent with the observation of specific viscosity (Fig. 2B). At high concentrations (≥ 1 g/L), the highly viscous suspensions exhibited a complex behavior upon shear rates. Consistent with previous findings (Kim *et al.*, 1999), these results confirmed the capacity of *M. xanthus* EPS to encase cells and form clumps in broth.

M. xanthus cell clumps formed in broth exhibit biofilm-like structures

CLSM in combination with specific indicator dyes was employed to *in situ* visualize the distributions of cells and EPS in the clumps or pellets. Counterstaining with SYTO 9 differentiated cells from debris and the matrix (Fig. 3, green). Respiring cells were visualized with CTC (Fig. 3, red). EPS was revealed by Alexa 633-WGA labeling (Fig. 3, blue). The pellets derived from 1 d cultures of strains with reduced (DK10410) or no (SW504 and SW810) EPS production, did not contain EPS-encased clumps but the cells were metabolically active (Fig. 3A and B). No respiring cells were apparent in the respective 12 d cultures (lacking of red staining, data not shown).

EPS-encased DK1622 (Wt) and DK3088 (*stk*) cell clumps appeared structurally similar to *M. xanthus* biofilms (Lux *et al.*, 2004) with respiring cells being embedded in the EPS network (Figs. 3C–F), especially in the clumps formed in 4 d broth (Figs. 3E and F). 12 d

cultures of DK1622 and DK3088 contained predominantly sphere-like cells that stained poorly with CTC (Fig. 3G) and were likely to be composed primarily of dead cells. However, these cultures also contained tight EPS matrix structures enclosing some metabolically active cells (Fig. 3H), which were observed more frequently in DK3088 (*stk*) cultures. The existence of these biofilm-like EPS structures might explain the longevity of DK3088 (*stk*) and DK1622 (Wt) under extended stationary phase culture conditions.

EPS-enclosed *M. xanthus* cells in clumps are more resistant to UV irradiation and SDS treatment

Since biofilm cells have a selective advantage for survival (Costerton *et al.*, 1995; Flemming and Wingender, 2010), response of EPS-enclosed *M. xanthus* clump cells to UV irradiation was examined. Planktonic cells of SW504 (Δ*difA*), SW810 (Δ*epsA*) and DK10410 (Δ*pilA*) exhibited similar resistance to UV light and required a similar dose for 99.9% of inactivation (Fig. 4, left panel). DK1622 (Wt) cells in clumps required an about 41 times higher dose for 99.9% inactivation, and DK3088 (*stk*) cells in clumps were the most UV-resistant cells. As expected, DK1622 planktonic cells collected from supernatant of broth were about 23 times less resistant than the clumping cells. Similar results were obtained for the sensitivity of cells to SDS (Fig. 4-right panel). The cells in DK1622 (Wt) and DK3088 (*stk*) clumps were more resistant to SDS-treatment than the planktonic cells. These results suggest that EPS matrix of clumps protects *M. xanthus* cells from environmental stresses.

EPS produced by wild-type cells have a protective effect on mutants deficient in EPS production

To further examine the protective role of EPS, an EPS^{-} strain, SW505, was co-cultured with DK1622 (Wt). SW505 is deficient in EPS production (Table 1) and carries a Tn5 insertion in the *difA* gene (Yang *et al.*, 1998), thereby allowing distinction from the kanamycin sensitive DK1622. After 12 d shaken, SW505 cultured alone did not survive, while the mixed culture still contained viable SW505 cells, which was comparable to the ratio of survival cells in the positive control (DK1622 alone). Furthermore, SW505 cells coexisting with DK1622-clumps in the mixed culture exhibited more resistance to UV irradiation and SDS treatment than the planktonic SW505 cells; they required about 11 times more UV dosage for 99.9% inactivation and showed about 25 times higher survival rate after SDS treatment (Fig. 4).

To determine whether SW505 (*difA*::Tn5) cells were included in DK1622 cell clumps, SW505 cells were inoculated with DK10547, a *gfp*-expressing derivative of DK1622 (Welch and Kaiser, 2001) with wild-type EPS production (Table 1). The SW505/DK10547 mixture from a one day culture was stained with SYTO 82 and Alexa 633-WGA (Fig. 5A). All the *gfp*-expressing DK10547 cells appeared green with compact red spots, since SYTO 82 bound the nucleic acids inside the cells. The aggregates were mainly composed of DK10547 cells (green with red) and EPS (blue), but also did contain SW505 cells (red only). In the clumps from DK10547 single culture, only green-red cells were observed after counterstaining (Fig. 5B). This clearly demonstrated the integration of SW505 into cell clumps with DK10547, which could be responsible for the increased survival rate and UV-resistance of the former.

Overproduced EPS traps live cells inside the matrix and prevents re-growth

While long-term survival under nutrient limitation is an important feature in the *M. xanthus* life-cycle, the ability to proliferate when environmental conditions improve is another important aspect. Therefore, we examined the ability of the EPS-producing strains to re-grow upon transfer into fresh medium after extended growth under stationary phase conditions. DK1622 clumps exhibited consistent re-growth, while no re-growth was

apparent for DK3088 cell clumps. However, after disassociation via vortexing, DK3088 cell clumps regained the ability of re-growth (Fig. 6A).

Given the apparent effect of EPS overproduction on re-growth of DK3088 (*stk*), we hypothesized that the over-abundance of EPS might reduce the recovery of *M. xanthus* cells from clumps. We examined the clump surface-medium interface of SYTO 9 (cells) and Alexa 633-WGA (EPS) labeled cell clumps with CLSM. Cultures that were able to re-grow had loosely packed EPS matrices that allowed the cells to be released (DK1622 cell clumps, Fig. 6B). On the other hand, cell clumps with densely packed EPS matrices, such as those present in the DK3088 4 d cultures, formed a thick layer of EPS at the interface between the cells and the medium (Fig. 6C). Thus, the cells in the overproduced-EPS matrix may be trapped, and unable to escape and proliferate.

Discussion

The biochemical analysis revealed that *M. xanthus* ECM was mainly composed of a carbohydrate matrix (EPS) with associated proteins (Behmlander and Dworkin, 1994; Dworkin, 1993). Further experiments have shown that the EPS portion is sufficient for rescuing social interactions in strains lacking ECM (Li *et al.*, 2003; Lu *et al.*, 2005). Consistent with these observations, the phenotypes of the mutants with alterations in different genetic loci responsible for *M. xanthus* EPS production examined in this study suggested that EPS was the key component for cellular agglutination and clumping in liquid cultures. This was also confirmed by examining the rheological properties of isolated EPS samples. For some bacteria, type IV pili (TFP) are part of the important extracellular apparatus for agglutination and biofilm formation (Costerton *et al.*, 1999). In *M. xanthus*, the cells lacking EPS (strain SW504, Δ*difA*) were defective in the formation of clumps or submerged biofilms (Yang, 2008) even when surface pili were overproduced (Li *et al.*, 2003). Cells lacking TFP (DK10410, Δ*pilA*) also failed to form clumps in liquid, which might be due to their much reduced EPS production regulated by TFP system (Black *et al.*, 2006) and viscosity. These observations suggested that the formation of clumps in *M. xanthus* was most likely correlated with EPS levels rather than the presence of surface pili.

For *M. xanthus* on solid surfaces, EPS is able to mediate cell-to-cell aggregation and adhesion to the growth substrata forming biofilms, preventing dehydration and protecting against phagocytosis and toxins (Dworkin, 1993). It has also been proposed that EPS could protect *M. xanthus* cells in their habitats by preventing toxic heavy metals from contacting the cells (Merroun *et al.*, 1998). In this study, we demonstrated that the EPS encased *M. xanthus* cell clumps in broth culture exhibited biofilm-like structures. In addition, physiologically like biofilm cells, the clumped cells have an increased capacity for stress resistance. For example, clumped cells exhibit prolonged cell viability in broth and increased resistance to both UV radiation and SDS treatment. The survival rate after UV irradiation of cells in the clumps was greater than that of free cells, which may be due to the physical shielding of cells by the EPS matrix or outer cells of clumps. Such a phenomenon was previously suggested in biofilms of *Pseudomonas aeruginosa* (Elasri and Miller, 1999). Indeed, biofilm cells are about 100 to 1000 times more resistant to UV than planktonic cells (Bak *et al.*, 2009). The increased resistance to the anionic surfactant SDS by clumped *M. xanthus* cells observed in this study also supports the idea that EPS could confer increased environmental resistance to biocides (Ganeshnarayan *et al.*, 2009; Mah and O'Toole, 2001). Our current findings are in agreement with the general observation that aggregates of bacterial cells (flocs or floccules) that are not attached to a surface share many characteristics with surface attached biofilms (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004). Considering that cells in biofilms express properties distinct from planktonic cells (Bhinu, 2005; Mah and O'Toole, 2001), *M. xanthus* cells may have different physiological

and metabolic properties when grown in clumps relative to suspensions and this may complicate their manipulation in laboratory settings.

Just as EPS underproduction is detrimental to survival, overproduction of EPS also appears to have a counterproductive long-term influence. Excess EPS production resulted in decreased viable cell recovery during extended late stationary phase. This may be partially attributable to the increased viscosity of the EPS obtained from the EPS-overproducing strain DK3088 (*stk*) as compared to WT-EPS suspensions. The cells in the overproduced-EPS matrix may be trapped, and unable to escape and proliferate. Therefore, optimal EPS production by *M. xanthus* is important for normal physiological functions.

Myxococcus ssp. is commonly found in terrestrial habitats (Dawid, 2000; Reichenbach, 1999), while fewer strains have been isolated from aqueous samples (Li *et al.*, 2002; Velicer and Hillesland, 2008). This observed difference in abundance between terrestrial and aquatic environments may be at least partially attributable to the fact that cells growing on terrestrial surfaces tend to produce more EPS for social behaviors, *e.g.* S motility (Li *et al.*, 2003; Lu *et al.*, 2005) and fruiting body development (Lux *et al.*, 2004; Shimkets, 1986). Our data suggests that many of these strains may be unable to exit their gelatinous EPS encapsulation when they are washed into aqueous environments. However, some *M. xanthus* strains have adapted to environments with periodic dry spells while also having the capacity to live permanently in fresh water habitats (Reichenbach, 1993). Forming biofilm-like clumps of cells in liquid, instead of living as dispersed planktonic cells, provides several selective advantages for *M. xanthus* in nature. The cells can prolong their viability inside clumps without developing fruiting bodies or myxospores, both of which require solid surface attachment (Diodati *et al.*, 2008). At the same time, it appears that EPS-encased *M. xanthus* cells in clumps are more resilient than planktonic cells in coping with environmental stresses like UV light.

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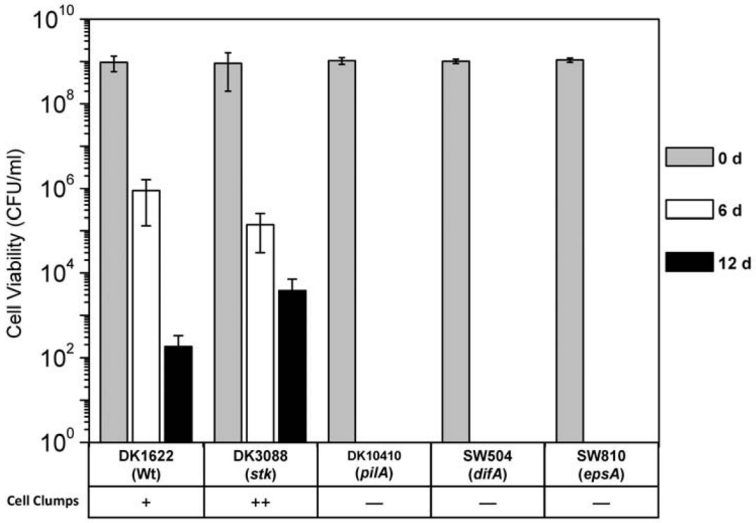

Fig. 1.

Viabilities of *M. xanthus* strains in liquid culture. Viable cells of different strains were enumerated from CFU per milliliter in broth on 0 d, 6 d and 12 d. The data represent triplicate experiments, and mean ± SD is plotted. Cell clumps in the liquid medium were examined through microscope and '−' represents not detected.

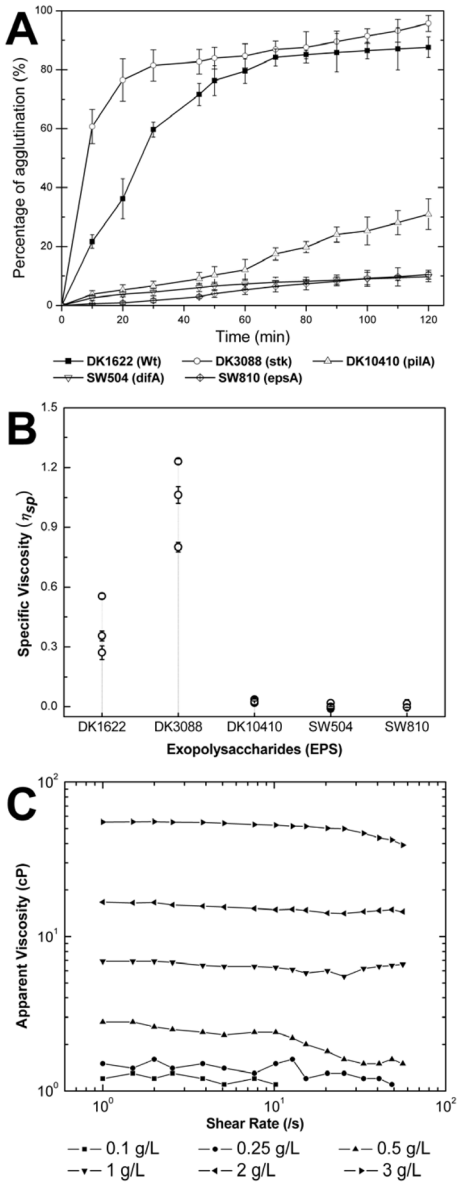

Fig. 2.

Agglutination of *M. xanthus* cells and rheological properties of EPS. Panel A: agglutination of mutants in broth. Cells were grown overnight in CYE medium and the OD_{600nm} was adjusted to about 1.0. The assay was conducted as described in *Materials and Methods*. The data represent triplicate experiments, and mean ± SD is plotted. Panel B: specific viscosity (η_{sp}) of EPS suspensions isolated from 5×10^{10} cells of different strains. For each strain, three parallel EPS samples were prepared and each sample was tested for three times, and mean ± SD were plotted. Panel C: influence of shear rate and concentration on the rheological curves of WT-EPS suspensions.

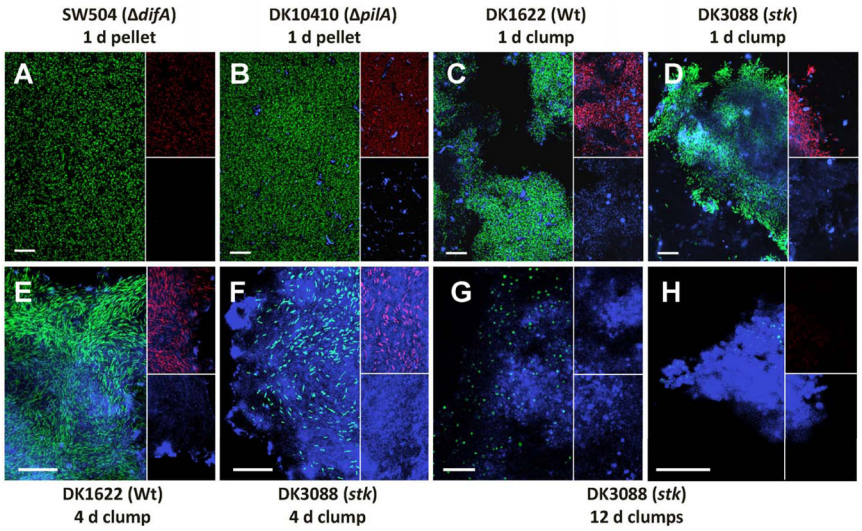

Fig. 3.

Examination of cells and EPS in cell pellets or clumps derived from different *M. xanthus* strains. Panels A and B: pellets of 1 d cultures of SW504 (Δ*difA*, and SW810-Δ*epsA* was similar in appearance) and DK10410 (Δ*pilA*) collected by centrifugation at 13,000 ×g for 5 min, respectively; panels C and D: clumps taken directly from 1 d cultures of DK1622 (Wt) and DK3088 (*stk*), respectively; panels E and F: clumps from 4 d cultures of DK1622 and DK3088, respectively; panels G and H: clumps from 12 d cultures of DK3088. Samples were counterstained with 5 μM SYTO 9, 5 mM CTC and 5 μg/ml Alexa 633-WGA in MOPS buffer. The panels are the overlay images of SYTO 9 (green) and Alexa 633-WGA (blue) signals, the upper small frames in the panels are the overlay images of CTC (red) and Alexa 633-WGA (blue) signals, and the lower frames are Alexa 633-WGA (blue) images, except that the upper small frame in the panel H shows the CTC (red) signal only. Bars represent 20 μm.

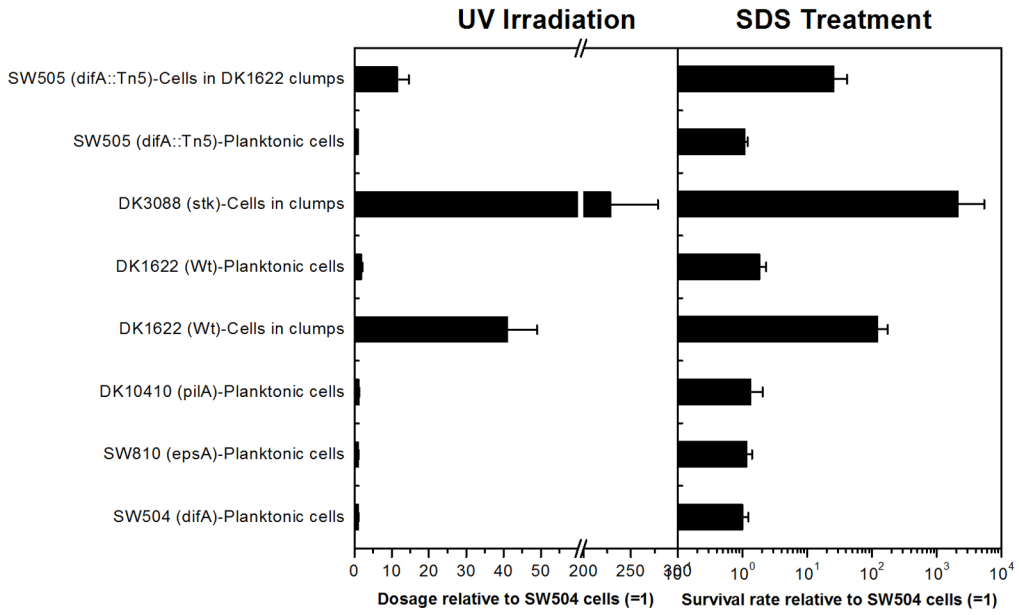

Fig. 4.

Stress survival of different *M. xanthus* strains. Left panel: relative UV_{254nm} doses required for 99.9% inactivation of different mutants compared to that for SW504 planktonic cells; right panel: relative survival rate after 0.01% SDS-30 min treatment of different cells compared to that of SW504 planktonic cells.

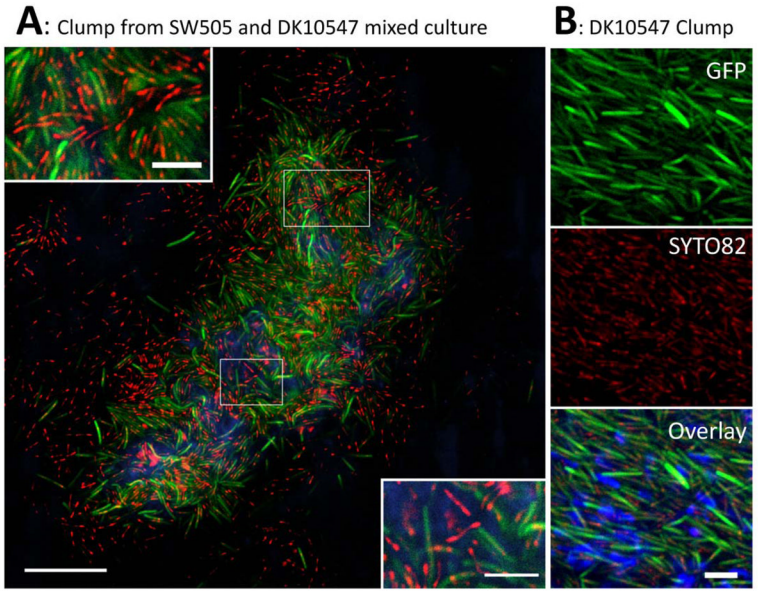

Fig. 5.

Aggregates of SW505 (*difA*::Tn5) cells, DK10547 (*gfp*-expressing derivative of DK1622) cells and EPS from a mixed 1 d culture (Panel A). Samples were counterstained with SYTO 82 and Alexa 633-WGA. The image is the overlay of *gfp*-expressing and SYTO 82 stained DK10547 (green with yellow dots), SYTO 82 stained SW505 (red) and Alexa 633-WGA-labeled EPS (blue) signals. Bar represents 20 μm. The small panels show magnified portions of the image indicated by the white panes and the bars represent 5 μm. Panel B, clump from a DK10547 (Gfp, upper small panel) single 1 d culture. Sample was counterstained with SYTO 82 (middle small panel) and Alexa 633-WGA (overlay with green and red signals in bottom small panel). Bar represents 5 μm.

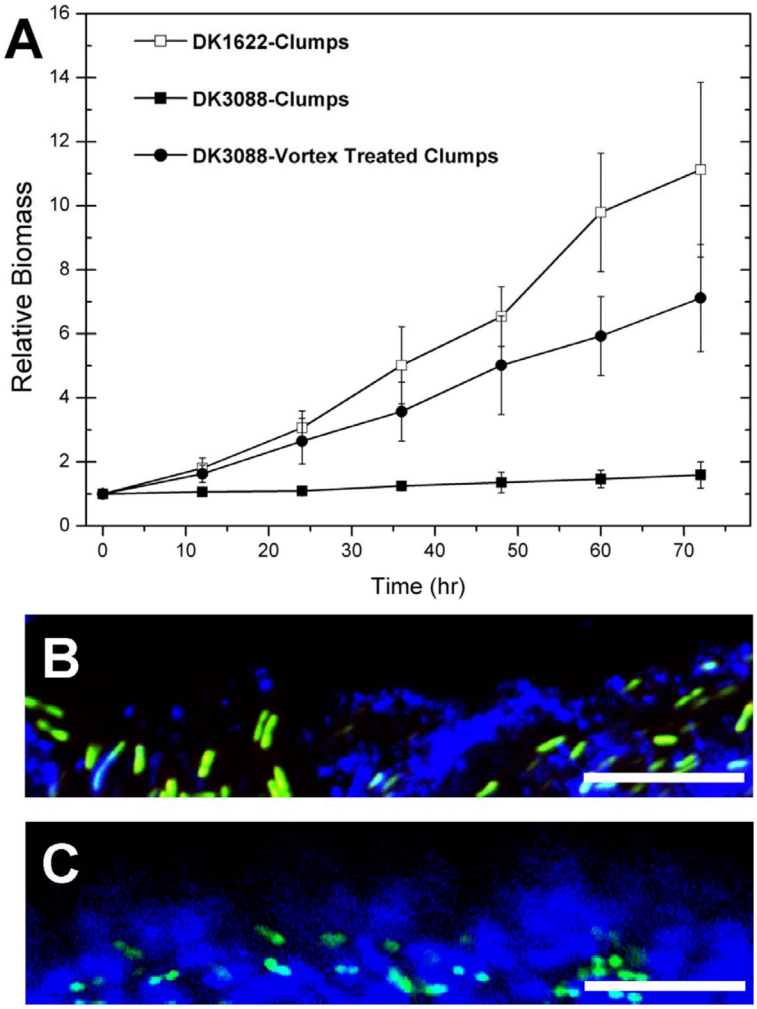

Fig. 6.

Re-growth and clump surface-medium interfaces of cell clumps of DK1622 (Wt) and DK3088 (*stk*). Panel A: re-growth curves of DK1622 and DK3088 (with or without pre-vortexing treatment) clumps form 4 d cultures. The relative biomass was calculated as dry biomass at each time point versus the dry biomass of inoculation (at 0 hr). Panels B and C show the clump surface-medium interfaces of different cell clumps examined through a 63× objective lens with CLSM and samples were counterstained with SYTO 9 (green) and Alexa 633-WGA (blue). Panels B is a clump from 4 d cultures of DK1622, and panels C is a clump from 4 d cultures of DK3088. Bars represent 10 μm.

Table 1

M. xanthus strains used in this study

Strain	Relevant genotype	EPS production[a]	Reference
M. xanthus			
DK1622	Wild type	100±9.3 %	(Kaiser, 1979)
DK10547	DK1622, *gfp*-expressing derivative	95.2±10.9 %	(Welch and Kaiser, 2001)
DK10410	DK1622, Δ*pilA*	21.5±5.6 %	(Wu and Kaiser, 1996)
DK3088	DK1622, *stk*	197.6±21.8 %	(Dana and Shimkets, 1993)
SW504	DK1622, Δ*difA*	2.7±1.6 %	(Yang *et al.*, 1998)
SW505	DK1622, *difA*::Tn5 kan903V101	3.2±1.9 %	(Yang *et al.*, 1998)
SW810	DK1622, Δ*epsA*	3.9±3.1 %	(Lu *et al.*, 2005)

[a]The data represent triplicate experiments, and mean ± SD is presented.