

RppA, a transducer homologue, and MmrA, a multidrug transporter homologue, are involved in the biogenesis and/or assembly of polysaccharide in *Myxococcus xanthus*

Yoshio Kimura, Saori Ishida, Hideki Matoba and Naoki Okahisa

Correspondence

Yoshio Kimura
kimura@ag.kagawa-u.ac.jp

Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-Cho, Kagawa 761-0795, Japan

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Myxococcus xanthus cells move by gliding, and form multicellular fruiting bodies under conditions of starvation. The authors cloned a gene, designated *rppA* (for receptor for polysaccharide production), which encodes a methyl-accepting protein homologous to the chemotaxis transducers in eubacteria. The *rppA* gene was co-transcribed with *mmrA*, a gene homologous to various multidrug transporter genes. The *rppA* or *mmrA* single mutants showed almost identical phenotypes to the wild-type strain; however, the *rppA*-*mmrA* double mutant exhibited reduced colony expansion, cell–cell agglutination and cellular reversal frequency. The double-mutant cells also showed less binding to Congo red, which mainly binds to fibril polysaccharide, than wild-type cells. Analysis of total polysaccharide in stationary-phase cells demonstrated that in the double mutant, polysaccharide levels were decreased by about 30% as compared with the wild-type strain. These results indicated that RppA and MmrA play a role in the biogenesis and/or assembly of polysaccharide, and the phenotypes of the double mutant may be due to the reduction in fibril polysaccharide.

INTRODUCTION

Myxococcus xanthus is a Gram-negative soil bacterium that moves by gliding (Hartzell & Youderian, 1995; Spormann, 1999; Wolgemuth *et al.*, 2002). The gliding motion of *M. xanthus* is controlled by two distinct multigene systems known as adventurous (A) motility and social (S) motility (Hodgkin & Kaiser, 1977a, b). A motility and S motility are required for the movement of single cells and cell groups, respectively. S motility is essential for cell movement in groups and during aggregation and fruiting-body formation. It requires at least the cell-surface components type IV pili and extracellular fibrillar material (Wu & Kaiser, 1995; Yang *et al.*, 1998, 2000). The type IV pili are localized predominantly at one cell pole and are thought to be the motors for the S motility of *M. xanthus* (Kaiser, 2000). The fibrils are constructed as carbohydrate structures with associated proteins, and are distributed over the cell surface (Behmlander & Dworkin, 1994). It is thought that fibrils function in cell–cell and cell–substratum contact (Arnold & Shimkets, 1988; Behmlander & Dworkin, 1991; Ramaswamy *et al.*, 1997). Li *et al.* (2003) proposed in a model for S

motility that *M. xanthus* extracellular fibril material interacts with pili and induces their retraction.

Chemotaxis transducers can sense changes in extracellular attractants and various repellents (Le Moual & Koshland, 1996; Parkinson, 1993). Typical chemotaxis transducers, known as methyl-accepting chemotaxis proteins (MCPs), are integral membrane proteins and have two distinct modules, an N-terminal periplasmic module involved in sensing various stimuli and a C-terminal cytoplasmic module involved in signalling and adaptation. Chemo-effector binding causes a conformational change in the cytoplasmic structure that regulates the activity of a two-component signal transduction system (a histidine kinase CheA and a response regulator CheY). In addition to these proteins, a linker (CheW) between CheA and MCP, a signal terminator (CheZ), a methyltransferase (CheR) and a methylesterase (CheB) are involved in the bacterial chemotaxis signal transduction pathway (Parkinson, 1993).

The *M. xanthus dif* locus encodes a set of chemotaxis homologues required for S motility and the biogenesis of fibrils (Yang *et al.*, 1998, 2000; Bellenger *et al.*, 2002). The *dif* mutants are defective in the formation of fruiting bodies, S motility, cellular cohesion and fibril biogenesis. Another chemotaxis locus, the *frz* locus, which also encodes chemotaxis homologues, is required for directed cell movement but not for S motility (Shi & Zusman, 1994; Ward &

Abbreviations: A motility, adventurous motility; S motility, social motility; MCP, methyl-accepting chemotaxis protein; PE, phosphatidylethanolamine.

The GenBank/DBJ accession numbers for the sequences reported in this paper are AB111917 (*rppA*) and AB111918 (*mmrA*).

Zusman, 1997). *frz* mutants show defects in the regulation of reversal in their gliding direction, and when starved they formed tangled frizzy filaments instead of normal fruiting bodies (Zusman, 1982; Blackhart & Zusman, 1985).

To study chemotaxis transducers, we cloned an MCP homologous gene, *rppA*, from an *M. xanthus* genomic library. The *rppA* gene formed an operon together with *mmrA*, homologous to various multidrug transporter genes. We report here that an *rppA*-*mmrA* double mutant of *M. xanthus* showed a phenotype similar to those of polysaccharide-reduced mutants.

METHODS

Bacterial strains and culture conditions. The type strain of *M. xanthus* (FB; = ATCC 25232, IFO 13542) was used as the wild-type (Dworkin, 1963) and was grown in CTT medium at 30 °C (Hodgkin & Kaiser, 1977). *M. xanthus* mutant strains were grown in CTT medium containing 100 µg kanamycin ml⁻¹. *Escherichia coli* LE392 (Promega) and NovaBlue (Novagen) were used as recipient strains in λ phage infection and plasmid transformation, respectively.

Cloning of *rppA* gene from a genomic DNA library. Two degenerate oligonucleotides (Hcd1, 5'-GG(C/G)TT(G/T)(G/T)C(C/G)GTGGTGGC(C/G)G(A/G)(C/G)GAG-3', and Hcd2, 5'-GG(C/G)-TTC(G/T)C(C/G)ATCGTGGC(C/G)G(A/G)(C/G)GAG-3') used as probes were designed to correspond to the highly conserved domain (HCD) of chemotaxis transducers (Le Moual & Koshland, 1996). The codon usage patterns of *M. xanthus* were used to minimize the overall degeneracy of each oligonucleotide. The oligonucleotides were labelled with digoxigenin-11-dUTP using an oligonucleotide tailing kit (Roche Diagnostics). The *M. xanthus* genomic library was constructed in λEMBL3 according to Kimura *et al.* (2001). A clone containing a putative transducer-encoding gene was isolated from the genome library by screening with a mixture of probes Hcd1 and Hcd2. The phage DNA of the clone was digested with *SacI* and ligated into the *SacI* site of pBluescript (Alting-Mees & Short, 1989) and sequenced by automated sequencing with the BigDye terminator kit (Applied Biosystems).

Construction of *rppA*, *mmrA* and *rppA*-*mmrA* disruption mutants. A 1.2 kb kanamycin-resistance cassette amplified by PCR from TnV (Furuichi *et al.*, 1985) was ligated into *NaeI*- and *StuI*-digested *rppA*, and into *NaeI*-digested *mmrA* (Fig. 1). The *rppA*::Km^r and *mmrA*::Km^r constructs were amplified by PCR using a pair of primers: 5'-TCGTGATGCTCGCATTTCG-3' (*rpp*-1) and 5'-GCATCAGCGTGGACATGC-3' (*mmr*-1), and 5'-TCAACGTCATCGACGACG-3' (*rpp*-2) and 5'-GCATCAGCGTGGACATGC-3'

(*mmr*-2), respectively. The *rppA*-*mmrA* double mutant was generated by insertion of the above kanamycin-resistance cassette into *NaeI*-digested *rppA* and *mmrA*. The *rppA*-*mmrA*::Km^r construct was also amplified by PCR using *rpp*-1 and *mmr*-2. The PCR products were used for electroporation, which was performed as described by Plamann *et al.* (1992). Gene replacement was verified by PCR and Southern hybridization analyses.

RT-PCR. Total RNAs were extracted from *M. xanthus* vegetative cells as described by Lee & Shimkets (1994) and treated with DNase I. One microgram of RNA was used for cDNA synthesis with *BcaBEST* polymerase in accordance with the manufacturer's protocol (Takara Shuzo). To determine whether *rppA*, *mmrA* and *orf1* formed an operon, PCR was performed with *Bca*-Optimized Taq polymerase using *rppA*-*mmrA* primers [forward primer (starting from the end region of the *rppA* gene), 5'-AGAAGGAGCAGACG-AAGG-3'; reverse primer (starting from the beginning region of the *mmrA* gene), 5'-TGTCACGAACTGGACCG-3'] and *mmrA*-*orf3* primers [forward primer (starting from the end region of the *mmrA* gene), 5'-GGCGGATTCTCACGCGTAG-3'; reverse primer (starting from the beginning region of *orf3*), 5'-CATTTCCTGACGCGCTCC-3']. Using *orf3* primers (forward primer, 5'-ATGGTGTTCATCCTGGTGGC-3'; reverse primer, 5'-GAGGTTCTCCGATTTCCTGG-3') for PCR, we also confirmed that *orf3* was transcribed in the *rppA*-*mmrA* mutant.

Development assay. Fruiting bodies were formed on CF plates (Hagen *et al.*, 1979) with 0.3% or 1.5% agar at 30 °C. The fruiting bodies were photographed under a light microscope. The fruiting bodies were harvested in TM buffer (10 mM Tris/HCl, pH 7.6, 8 mM MgCl₂), and sonicated using a Branson sonifier. After incubation for 2 h at 50 °C, the number of viable spores was quantified by plating on CYE agar (Campos *et al.*, 1978).

Colony spreading and agglutination assays. For colony spreading assays, 2 µl *M. xanthus* cells at 1 × 10¹⁰ cells ml⁻¹ was spotted onto the centre of CYE medium with 0.3% or 1.5% agar, and incubated at 32 °C for 4 days (Shi & Zusman, 1993). The agglutination assay was performed as described by Kim *et al.* (1999). Cells from exponential cultures were washed twice with 10 mM MOPS (pH 6.8), inoculated into MCM buffer (10 mM MOPS, pH 6.8, 10 mM MgCl₂, 1 mM CaCl₂) or CYE medium to a density of 2 × 10⁸ cells ml⁻¹, and incubated statically at 25 °C. At 10 or 30 min intervals, the OD₆₀₀ was measured in a spectrophotometer.

Congo red binding assay. The wild-type and mutant cell suspensions, at a density of 1 × 10⁹ cells ml⁻¹, were placed on TPM agar containing 57 µM Congo red, and incubated at 30 °C for 24 h (Kearns *et al.*, 2002). The colour of the cell aggregates was observed.

Reversal period assay. This assay was performed by a modification of the method previously described by Kearns *et al.* (2000).

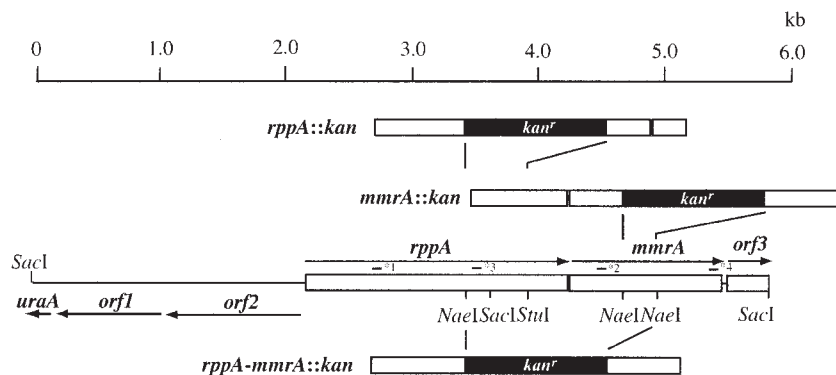


Fig. 1. Restriction map of the *rppA* and *mmrA* genes of *M. xanthus*. Arrows indicate orientation. This map also shows the location of the kanamycin-resistance gene (Km^r) inserted in *rppA*, *mmrA* and *rppA*-*mmrA*. *1, *2, *3, and *4 indicate the location of *rpp*-1, *mmr*-1, *rpp*-2 and *mmr*-2 primers, respectively.

Dilauroyl phosphatidylethanolamine (PE) and dioleoyl PE were dissolved in chloroform and chloroform/methanol (1:1, v/v), respectively. Four microlitres of PE solution ($0.05 \mu\text{g } \mu\text{l}^{-1}$) was applied to TPM agar, and dried for 20 min at 37°C . Five microlitres of *M. xanthus* cells diluted to 2×10^7 cells ml^{-1} in MOPS buffer (10 mM MOPS, pH 7.6, 8 mM MgSO_4) was inoculated on top of the test compound and incubated at 30°C for 20 min. The cellular reversal frequency was monitored with a digital camera through a Nikon microscope. At least 20 cells from each strain were followed over a 45 min period.

Western blotting analysis of fibrils. *M. xanthus* wild-type and mutant cells were incubated in MCM buffer for 5 h at room temperature, harvested and washed with 10 mM MOPS buffer (pH 7.5). Proteins of cells (5×10^8) were separated by SDS-PAGE (12% polyacrylamide) and electroblotted onto PVDF membrane (Bio-Rad) using a Trans Blot SD semidry transfer cell (Bio-Rad) according to the manufacturer's instructions. The membranes were blocked with 3% bovine serum albumin in PBS-T buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.1% Tween 20) and then developed with a primary antibody mAb 2105 (Behmlander & Dworkin, 1991) and a secondary antibody composed of horseradish-peroxidase-conjugated goat anti-mouse immunoglobulin G. The membranes were washed with PBS-T buffer and detected with ECL Western blotting detection reagents (Amersham Biotech).

Polysaccharide measurement. *M. xanthus* polysaccharide was measured by a modification of a method described previously (Kim *et al.*, 1999). *M. xanthus* cells grown in CYE medium to a density of 5×10^8 cells ml^{-1} were collected by centrifugation, and resuspended at 2×10^9 cells ml^{-1} in fresh CYE medium. Samples were collected from the cell suspensions at the beginning and end of a 6 h incubation, washed in 0.85% NaCl, and sonicated. The pelletable carbohydrate was obtained by centrifugation, and used to measure polysaccharide. Protein concentration was determined using the protein assay of Bradford (1976).

RESULTS

Cloning and amino acid sequence homology

In the search for transducer genes, two oligonucleotide probes were synthesized based on eight amino acids [GFAVVA(D/E/G)E] from the highly conserved domain (HCD) of chemotaxis transducers. One positive clone that hybridized strongly with the probes was obtained from the *M. xanthus* genomic library in λ EMBL3. We sequenced various DNA restriction fragments of the clone and determined that the 3.6 and 2.2 kb *SacI* fragments contained four complete open reading frames (*orf1*, *orf2*, *rppA* and *mmrA*) and two incomplete open reading frames (*uraA* and *orf3*) (Fig. 1). The *rppA*, *mmrA* and *orf3* genes are oriented in the same direction.

The nucleotide sequence of *rppA* (GenBank accession no. AB111917) encodes a polypeptide composed of 718 amino acids corresponding to a molecular mass of 77 682 Da. The C-terminal region of the deduced protein from *rppA* shows very strong similarity to the chemotaxis transducer proteins, and shares homology with domains in the PilJ protein of *Pseudomonas aeruginosa* (29% identity, 54% similarity) (Darzins, 1994), the NahY protein of *Pseudomonas putida* (30% identity, 46% similarity) (Grimm &

Harwood, 1999) and the DifA protein of *M. xanthus* (24% identity, 47% similarity) (Yang *et al.*, 1998). The putative RppA protein possesses four transmembrane helices (residues 22–39, 49–69, 169–189, and 199–219), a HAMP (linker) domain (residues 221–273), at least two methylation domains (residues 385–399 and 609–624), a signalling domain (residues 372–633), and a coiled coil (residues 682–718) (Zhulin, 2001). Four repeated sequences (EQA/TXGSK/EQV/IXXS/AXE/H), partially similar to the methylation domain, were present in the C-terminal region (residues 582–679) of RppA.

The *mmrA* gene (GenBank accession no. AB111918) was located 11 bp downstream from the stop codon of the *rppA* coding region. The predicted MmrA protein was 425 amino acids in length with a molecular mass of 44 689 Da. The *mmrA* gene product exhibited similarity to multidrug transporters; it exhibited sequence similarity with the putative multidrug-resistance protein (Mdr) of *Aeromonas hydrophila* (33% identity, 54% similarity) (Zhang *et al.*, 2000), efflux pump protein (MxcK) of *Stigmatella aurantiaca* (35% identity, 52% similarity) (Silakowski *et al.*, 2000) and chloramphenicol-resistance protein (Cmr) of *Rhodococcus fascians* (25% identity, 40% similarity) (Desomer *et al.*, 1992). MmrA is predicted to consist of 12 transmembrane α -helices and has several highly homologous amino acid sequence motifs (GXXXDRXGRR in loops 2–3 and 8–9, and DXXXXXXR in loop 4–5) (Rouch *et al.*, 1990; Yamaguchi *et al.*, 1992) that are conserved in equivalent positions of proton-coupled symporters, antiporters and uniporters. The *orf3* gene encodes a Tat translocase homologue (Sargent *et al.*, 1998), and it was located 53 bp downstream of *mmrA*.

We examined whether *rppA*, *mmrA* and *orf3* were co-transcribed in the wild-type strain using RT-PCR. RT-PCR, with primers that amplified a 247 bp fragment of *rppA*-*mmrA*, or a 155 bp fragment of *mmrA*-*orf3*. The results of these experiments suggested that *rppA* and *mmrA* were transcribed as a single transcript, but that *orf3* was not co-transcribed with *rppA* and *mmrA* (Fig. 2). We also examined whether *orf3* was transcribed in the *rppA*-*mmrA* mutant. The expected 169 bp product was amplified from total RNA of the double mutant when treated by reverse transcriptase (Fig. 2), indicating that *orf3* was expressed in this mutant. We confirmed that the RT-PCR products were detected at the same positions as PCR products amplified from genomic DNA on agarose gel (data not shown).

The *uraA* and *orf1* genes have been described previously in *M. xanthus* and were shown to encode an orotidine-5'-monophosphate decarboxylase and a hypothetical oxidoreductase, respectively (Kimsey & Kaiser, 1992). The deduced amino acid sequence of *orf2* exhibited high similarity to that of human histone deacetylase 6.

Characterization of mutants

Construction of mutants. A specific *rppA* or *mmrA* deletion mutant was constructed using the gene replacement

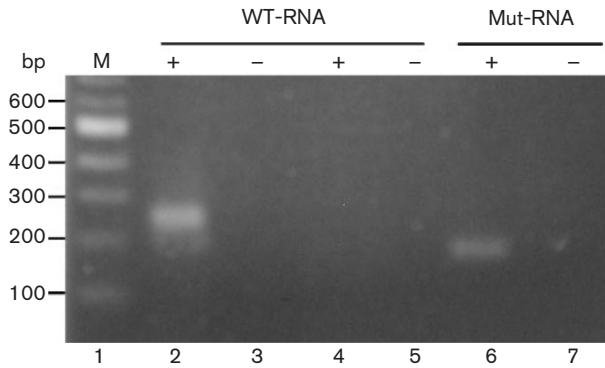


Fig. 2. Co-transcription of *rppA* and *mmrA* in the wild-type strain and *orf3* transcript detection in the double mutant strain. Total RNAs prepared from vegetative cells of the wild-type (lanes 2–5) and the *rppA-mmra* mutant (lanes 6 and 7) were either treated (lanes 2, 4 and 6) or not treated (lanes 3, 5 and 7) with reverse transcriptase. PCRs were performed using *rppA-mmra* primers (lanes 2 and 3), *mmrA-orf3* primers (lanes 4 and 5), and *orf3* primers (lane 6 and 7) as described in Methods. M (lane 1), molecular mass standards.

technique (Fig. 1). In *rppA* and *mmrA* mutants, the *NaeI*–*StuI* fragment of the *rppA* gene comprising the signalling and R1 domains, and the middle region (*NaeI* fragment) of the *mmrA* gene, were removed and replaced with kanamycin-resistance cassettes. An *rppA-mmra* double mutant, which lacked the signalling and RI domains of RppA and the amino-terminal half of MmrA, was also constructed and examined with various phenotypic tests.

Developmental phenotype. The mutants were examined for fruiting body development. The wild-type strain and all mutant strains formed normal fruiting bodies after 2–3 days of incubation on CF plates with 1.5% agar. Within the fruiting bodies of the wild-type and *mmrA* mutant, vegetative cells were converted into spherical myxospores, while the *rppA* and *rppA-mmra* mutants formed many short rod-shaped spores. The double mutant cells formed fruiting bodies about 1 day later than the wild-type strain, and the viable spore yield of the double-mutant strain was approximately 30% of that of the wild-type strain (Fig. 3a). There were no obvious differences in viable spore yields between wild-type, *rppA* and *mmrA* mutants (data not shown). When spotted on CF plates with 0.3% agar, the wild-type, *rppA* and *mmrA* mutants formed fruiting bodies, but the *rppA-mmra* mutant did not (Fig. 3b).

Colony spreading. To analyse the effect of the *rppA* mutation on cell motility, the rate of colony spreading of mutant cells on CYE plates containing 0.3% or 1.5% agar was compared with that of the wild-type strain. The colony expansion of the *M. xanthus* type culture strain (wild-type) cells used in this study was much slower than

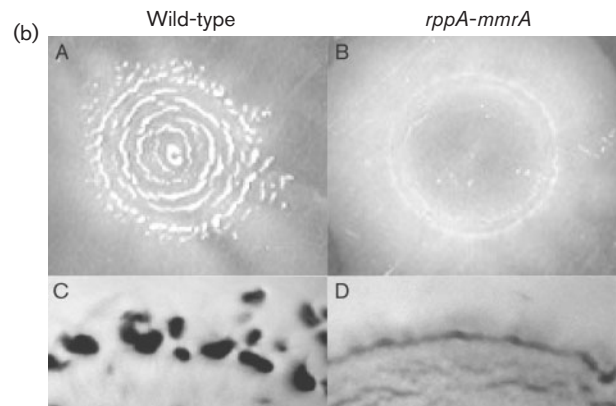
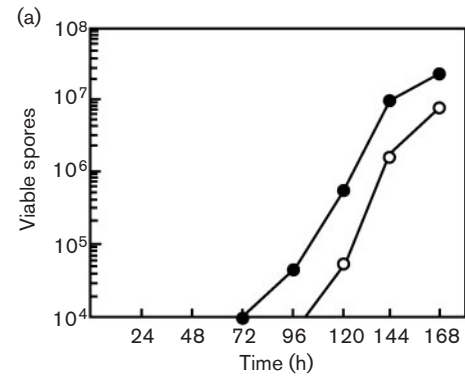


Fig. 3. (a) Viable spore formation of *M. xanthus* wild-type (●) and the *rppA-mmra* mutant (○) incubated on CF plates with 1.5% agar. (b) Photographs of fruiting bodies on CF plates with 0.3% agar, taken 7 days after the start of development. The photographs are shown at low ($\times 8$; A, B) and high ($\times 4$; C, D) magnification.

that of *M. xanthus* DK1622 (wild-type) cells (Shi & Zusman, 1993; Ramaswamy *et al.*, 1997; Yang *et al.*, 1998). The *rppA* and *mmrA* single mutants both showed similar colony spreading to the wild-type. In contrast, *rppA-mmra* double-mutant cells on 1.5% agar appeared to spread more slowly than wild-type cells (Fig. 4a, b). The rate of colony expansion of the double mutant was reduced to 60–70% of the parental wild-type level in 4 days. The reduced colony expansion of the double mutant cells was complemented by mixing with wild-type cells in a ratio of 3:1 (mutant cells/wild cells) (data not shown). Similar to the wild-type strain, the double-mutant cells on 1.5% agar had a rough colony edge with both single cells and rafts of cells, suggesting that the double mutant demonstrated both A and S motility (data not shown). It was reported that *M. xanthus* DK1622 and A^-S^+ motility mutant cells form much larger swarming colonies on 0.3% agar at 3 days of incubation (Shi & Zusman, 1993). The colony spreading of wild-type used in this study was about the same on both 0.3% and 1.5% agar during the first 2 days of incubation, and then the wild-type cells spread more on 1.5% agar (Fig. 4a, c).

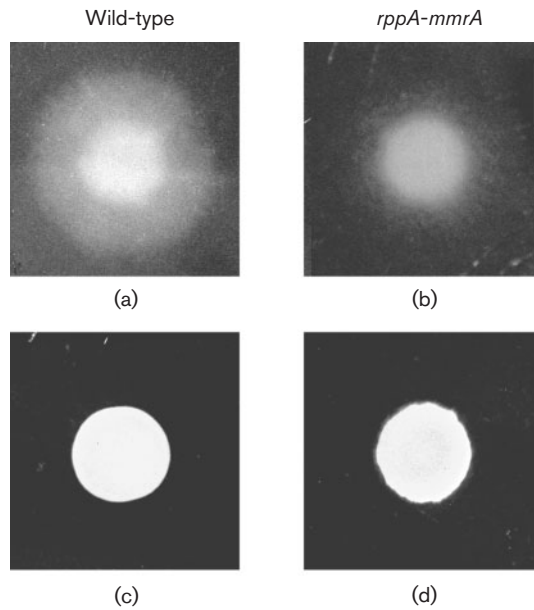


Fig. 4. Colony spreading of wild-type and *rppA-mmra* mutant strains on CYE plates containing 1.5% agar (top) or 0.3% agar (bottom). Photographs were taken after 4 days of incubation. The initial size of the colonies was 0.3–0.4 cm. The diameters of the colonies as photographed were: (a) 1.2, (b) 0.8, (c) 0.6 and (d) 0.6 cm.

On 0.3% agar, the wild-type and *rppA-mmra* mutant cells formed colonies similar in size (Fig. 4c, d).

Cell agglutination. Cellular cohesion is closely correlated with fibril induction and S motility (Yang *et al.*, 1998). In agglutination buffer, wild-type, *rppA* and *mmra* mutant cells began to agglutinate after 40 min of incubation, and were precipitated within 120 min (Fig. 5). In contrast, the *rppA-mmra* mutant cells agglutinated more slowly than wild-type cells, and were not completely precipitated after 200 min of incubation. Congo red acts as a specific inhibitor of S motility (Arnold & Shimkets, 1988). Addition of Congo red to the agglutination buffer completely inhibited the agglutination of wild-type and double-mutant cells (data not shown). The double-mutant cells also showed delayed agglutination in CYE medium, whereas the wild-type and single mutants agglutinated normally (data not shown).

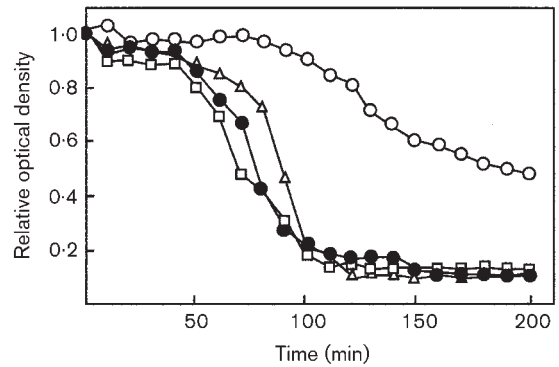


Fig. 5. Cellular agglutination of wild-type (●), *rppA* (Δ), *mmra* (□), and *rppA-mmra* (○) mutant strains. The relative optical density was calculated by dividing the OD₆₀₀ value at a given time by the initial OD₆₀₀ of the sample.

Reversal frequency. The cellular reversal of *M. xanthus* gliding is regulated by chemotaxis homologues (Shi *et al.*, 2000). The wild-type strain used in this study showed a shorter basal reversal period (about 3.5 min) as compared to wild-type DK1622 (about 5–7 min) (Blackhart & Zusman, 1985; Spormann & Kaiser, 1999). The double mutant had an increased (1.4-fold) basal reversal period when observed on CYE or TPM medium. Wild-type cells usually moved in the direction of the long axis of the cell, but as shown in Fig. 6, the double-mutant cells frequently had bent bodies, and changed their direction of movement, especially when observed on TPM medium. The lipid PE, especially dilauroyl (di C12:0) and dioleoyl (di C18:1 ω 9c) PE, regulates the cell reversal frequency of *M. xanthus* cells (Kearns *et al.*, 2000). The reversal period assay was performed on TPM medium with 0.2 μ g dilauroyl PE or dioleoyl PE; the double-mutant cells showed reduced excitation in response to dilauroyl PE but not to dioleoyl PE. Dioleoyl PE lengthened the reversal period of the mutant cells from 5.0 min to 8.6 min. This phenotype is similar to that of mutants for *difA*, an MCP homologue, and *fibA*, a matrix-associated zinc metalloprotease homologue called fibril protein A (Kearns *et al.*, 2000, 2002).

Congo red binding assay. The diazo dye Congo red and the fluorescent dye calcofluor white bind to fibril polysaccharide of *M. xanthus* (Behmlander & Dworkin, 1991;

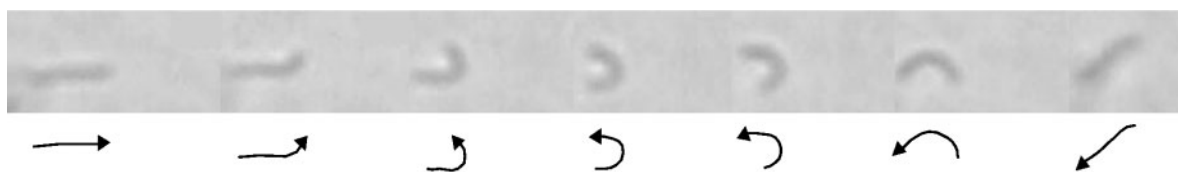


Fig. 6. Movement of *rppA-mmra* mutant cells on TPM agar. The mutant cells frequently bent into a U shape, and changed their direction of movement. The arrows indicate the direction of cell movement.

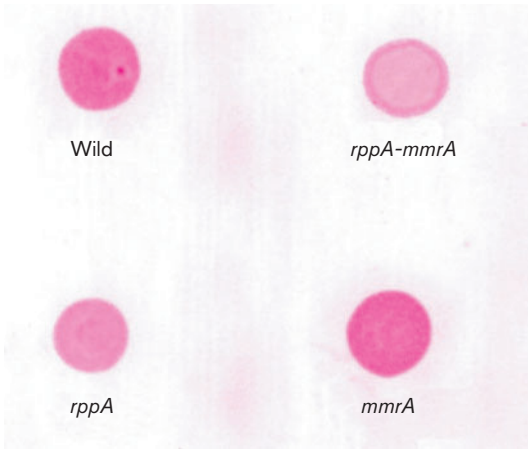


Fig. 7. Congo red binding by wild-type and mutant strains incubated on TPM agar containing Congo red for 24 h.

Ramaswamy *et al.*, 1997). When wild-type and mutant cells were placed on TPM agar containing Congo red for 24 h, cell aggregates of the wild-type, *rppA* mutant and *mmrA* mutant turned red, whereas cell aggregates of the double mutant turned light pink (Fig. 7), indicating that the double-mutant cells bound the dye poorly compared to wild-type cells. On the other hand, when cultured on CYE medium containing calcofluor white ($50 \mu\text{g ml}^{-1}$), neither the wild-type nor the mutant cells showed obvious binding of the dye.

Analysis of polysaccharide and fibril protein. *M. xanthus* fibrils contain a large amount of protein and polysaccharide, and the biogenesis of fibrils is induced under stationary-phase or starvation conditions (Arnold & Shimkets, 1988; Behmlander & Dworkin, 1991). The *rppA-mmra* mutant cells in exponential phase produced less polysaccharide than wild-type cells, and there was a reduced induction of polysaccharide production in the stationary phase (Table 1). There was no significant difference in the amount of fibril-specific protein between

Table 1. Polysaccharide content of wild-type and mutant strains

Samples from exponential- and stationary-phase cells were tested. The values are the means of measurements from three independent experiments \pm SE.

Strain	Carbohydrate content [$\mu\text{g carbohydrate (mg protein)}^{-1}$]	
	Exponential	Stationary
Wild-type	57.8 ± 12	94.6 ± 5
<i>rppA</i>	53.0 ± 8	80.0 ± 2
<i>mmrA</i>	60.0 ± 9	92.1 ± 12
<i>rppA-mmra</i>	49.4 ± 6	51.0 ± 2

wild-type and mutant strains in the immunoblot analysis (data not shown).

DISCUSSION

In *M. xanthus*, there are two chemosensory systems, *frz* and *dif*, both of which are homologous to known chemotaxis proteins from enteric bacteria that have been well characterized (McBride *et al.*, 1989). The *frz* and *dif* loci encode at least five genes homologous to the enteric chemotaxis proteins. Recently, a third chemotaxis-like gene cluster was identified; this cluster encodes at least six chemotaxis genes or homologues (Kirby & Zusman, 2003). In this study, a gene homologous to the MCP gene, *rppA*, was identified, but the two *SacI* fragments containing *rppA* did not encode other chemotaxis proteins. Therefore we sequenced further a region of about 4 kb downstream from the *mmrA* gene, but this did not identify any other chemotaxis genes. Typical chemotaxis transducer proteins consist of a ligand-binding periplasmic domain, and a cytoplasmic region including a linker, methylation domains and a signalling domain. RppA does not have a large periplasmic region composed of over 100 amino acids, but instead has two very short periplasmic spans (each about 10 amino acids) between the transmembrane regions in the N-terminal periplasmic domain. This structural feature is found in DifA of *M. xanthus* and HtrI of *Halobacterium salinarum* (Seidel *et al.*, 1995), which are class III MCPs as described by Zhulin (2001).

The *rppA-mmra* mutant showed reduced motility on 1.5% agar and reduced cellular cohesion in agglutination buffer. In addition, the mutant had a longer basal reversal period, and reduced excitation in the reversal period in response to dilauroyl PE. On the other hand, *rppA* and *mmrA* single mutants showed wild-type phenotypes except for the spore shape of the *rppA* mutant. Since we confirmed, using RT-PCR, that *orf3*, located downstream of *mmrA*, was transcribed in the *rppA-mmra* mutant, the phenotypes of the double mutant would not be due to polar effects. These results imply that the single deletion of RppA or MmrA may be complemented by other MCPs or multidrug transporters, respectively; however, the double deletion may make complementation with these proteins difficult. We searched the *M. xanthus* genome sequence database (TIGR database) for genes that encode proteins homologous to RppA or MmrA. The two gene regions with highest homology to RppA and MmrA were located at sequence positions 212732–214165 and 2845621–2846667, respectively; the proteins encoded by these two gene regions showed 26% identity and 45% similarity to RppA and 35% identity and 55% similarity to MmrA.

The phenotypes of the double mutant show some similarity to those of fibril-deficient mutants (Kearns *et al.*, 2000, 2002). *M. xanthus* fibrils are used to attach to other cells, and are required for cell–cell cohesion and S motility. Since fibril-deficient mutants (*tglA/stk*, *dsp* and *difA*) or

a fibril protein A deficient mutant (*fibA*) lack excitation in response to dilauroyl PE, it has been thought that fibrils contain the dilauroyl PE chemoreceptors. The *difA* mutant, completely lacking fibril protein and polysaccharide, showed no obvious signs of agglutination, and was defective in S motility (Yang *et al.*, 2000). However, the *rppA-mmra* mutant was not completely deficient in swarming and cohesion, and had A and S motility cells at the colony edge, suggesting that the double-mutant cells can form fibrils but that the amount of fibril may be lower than that in wild-type cells. We next compared the amount of polysaccharide and fibril protein in the wild-type and the mutant cells. Immunoblot analysis showed no obvious difference in fibril-specific protein between wild-type cells and the mutant cells. On the other hand, the double-mutant cells had reduced amounts of polysaccharide compared with wild-type cells. This result was confirmed by the fact that the double-mutant cells were not well stained with Congo red. The phenotypes of the double mutant may be due to the reduction in fibril polysaccharide. This phenotype is similar to that of the calcofluor white binding deficient mutants (Cds) of *M. xanthus* (Ramaswamy *et al.*, 1997). Nine Cds mutants generated by transposon mutagenesis showed significantly lower levels of stationary-phase polysaccharide than the wild-type strain. The Cds mutants also had decreased cell cohesion, fibril production and fruiting body formation. There is a possibility that the transposon insertions in one of or some Cds mutants may have occurred in the *rppA* gene. In recent years, it has become clear that there is a relation between cell motility, colony morphology and polysaccharide production in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Vibrio parahaemolyticus* (D'Argenio *et al.*, 2002; Guvener & McCarter, 2003; Spiers *et al.*, 2003).

Sequence analyses suggest that MmrA is a potential member of the major facilitator superfamily (MFS) of transporters. Several transporters involved with bacterial chemotaxis have been reported. The *M. xanthus* ATP-binding cassette (ABC) transporter, AbcA, was found to interact with FrzZ, a CheY homologue protein, using the yeast two-hybrid system (Ward *et al.*, 1998). Ward *et al.* (1998) speculated that AbcA might be involved in the export of a molecule required for the autochemotactic process. *M. xanthus pilH* encodes an ABC transporter homologue that is required for type IV pilus biogenesis, and a *pilH* mutant lacks S-motility (Wu *et al.*, 1998). Tol proteins, known to be involved in macromolecule transport, were recently shown to be required for *M. xanthus* A motility (Youderian *et al.*, 2003). *Pseudomonas putida* PcaK is an MFS transporter and chemoreceptor protein that functions in aromatic acid chemotaxis (Harwood *et al.*, 1994). Although a single mutation in the *mmra* gene did not affect the phenotype, the *rppA-mmra* double mutation appeared to decrease the amount of polysaccharide in stationary-phase cells. MmrA and RppA may interact and function in the biogenesis and/or assembly of polysaccharide. In contrast to *dif* mutants, the *rppA-mmra*

mutant was not completely deficient in polysaccharide. It is also possible that RppA and MmrA proteins have other functions. We are currently investigating whether these proteins possess other functions in *M. xanthus*.

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