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Killing of *Escherichia coli* by *Myxococcus xanthus* in Aqueous Environments Requires Exopolysaccharide-dependent Physical Contact

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Abstract

Nutrient or niche-based competition among bacteria is a widespread phenomenon in natural environment. Such inter-species interactions are often mediated by secreted soluble factors and/or direct cell–cell contact. As ubiquitous soil bacteria, *Myxococcus* species are able to produce a variety of bioactive secondary metabolites to inhibit the growth of other competing bacterial species. Meanwhile, *Myxococcus sp.* also exhibit sophisticated predatory behavior, an extreme form of competition that is often stimulated by close contact with prey cells and largely depends on the availability of solid surfaces. *Myxococcus sp.* can also be isolated from aquatic environments. However, studies focusing on the interaction between *Myxococcus* and other bacteria in such environments are still limited. In this study, using the well-studied *M. xanthus* DK1622 and *E. coli* as model interspecies interaction pair, we demonstrated that in a aqueous environment, *Myxococcus xanthus* was able to kill *Escherichia coli* in a cell contact-dependent manner, and that the observed contact dependent killing required the formation of co-aggregates between *M. xanthus* and *E. coli* cells. Further analysis revealed that exopolysaccharide (EPS), type IV pilus (TFP) and lipopolysaccharide (LPS) mutants of *M. xanthus* displayed various degrees of attenuation in *E. coli* killing, and it correlated well with the mutants' reduction in EPS production. In addition, *M. xanthus* showed differential binding ability to different bacteria, and bacterial strains unable to co-aggregate with *M. xanthus* can escape the killing, suggesting the specific nature of co-aggregation and the targeted killing of interacting bacteria. In conclusion, our results demonstrated EPS mediated, contact-dependent killing of *E. coli* by *M. xanthus*, a strategy that might facilitate the survival of this ubiquitous bacterium in aquatic environments.

Keywords

M. xanthus; contact-dependent interaction; co-aggregation; aqueous environment

Introduction

Cooperation and competition within and between bacterial species to enhance their survival in natural environments is a common theme in microbial ecology [1, 2]. For example, metabolic cooperation among different bacteria species is essential for obtaining nutrients and the maintenance of the bacterial consortia [3, 4]. Meanwhile, nutrient or niche-based competition is a more widespread phenomenon among different bacterial species within

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natural microbial communities [5]. During competition, bacteria often employ different strategies, such as producing antibiotics or toxic secondary metabolites to inhibit or kill the competing microbial species, thus gaining growth advantage [6, 7]. One of the extreme forms of competition is predation, where predatory bacterial species kill and lyse their targeted prey cells to obtain cellular components as nutrients needed to sustain their growth [8, 9].

M. xanthus, the model species of *Myxococcus*, is an ubiquitous soil bacterium that competes with other microbial species by producing a slew of secondary metabolites to inhibit their growth. More importantly, on solid surfaces, *M. xanthus* demonstrates several complex social behaviors, such as social motility, fruiting body formation, and particularly, predation behavior [10-13], a competition strategy that is often stimulated by close contact with prey cells and enables *M. xanthus* to prey upon other bacteria for better survival. The detection of prey cells, as well as the coordinated release of bacteriolytic enzymes, proteases, and antibiotics for prey killing require extensive and well-regulated intra- and interspecies interactions.

Although *Myxococcus* sp. are commonly found in various types of terrestrial habitats, many of them, including *M. fulvus*, *M. visrescens*, *M. xanthus* etc., can also be frequently isolated from many aquatic environments [14-16], presumably as a result of soil organism being regularly washed into the water and surviving there [13, 17, 18]. As flagella-less bacteria, they are non-motile and often lose solid surface dependent social behaviors under liquid conditions [19] and little is known regarding their strategies to compete with other bacteria to survive in aqueous environments. The most frequent places one can isolated myxobacteria is animal dung, which is full of *E. coli* cells. Thus the *M. xanthus* and *E. coli* is a well-known model system to study interspecies interaction in the field of myxobacteria [17]. In this study, by using the well studied *M. xanthus* DK1622 and *E. coli* MG1655 as model microorganisms, we aimed to study the interspecies interaction between these two species in aquatic environments. The killing of *E. coli* by *M. xanthus* under aqueous condition was analyzed, and the cell components involved in interspecies interaction were further investigated.

Materials and methods

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1. *E. coli* strains from the Large-scale Chromosome Deletion and *E. coli* Transposon Insertion Mutation Libraries (NBRP, National Institute of Genetics, Japan), *Klebsiella pneumoniae* IA565 [20], *Pseudomonas aeruginosa* PA01 and *Micrococcus luteus* were cultured in liquid Luria-Bertani (LB) broth or on solid LB containing 1.5% agar. The *M. xanthus* strains were cultured in CYE liquid medium at 32 °C on a rotary shaker at 300 rpm or on CYE plates containing 1.5% agar (Difco) [21]. When needed, kanamycin (Km, 100 µg/ml) was added to the media.

Two Chamber Assay

The two-chamber assay was carried out as reported [22, 23] with some modifications. *M. xanthus* and *E. coli* strains were grown to the exponential phase, harvested, and re-suspended to OD₆₀₀ of 1 in fresh CYE medium. The *M. xanthus* cells alone or together with *E. coli* cells were inoculated into the bottom chamber of a 12-well plate containing a 0.4-µm PET membrane insert (Millipore, Billerica, MA, USA), while wild-type *E. coli* was added to the top chamber. Meanwhile, the mixed *M. xanthus* - *E. coli* cells as well as *E. coli* cells alone were inoculated into the 12-well plate without the top chamber, serving as controls. The culture condition for this assay was at 32 °C on a rotary shaker at 130 rpm. The *M.*

xanthus-to-*E. coli* ratio for the two-chamber assay was 100:1. Three replicates were performed for each experiment.

Trypan Blue Binding Assay

To monitor EPS production of the selected *M. xanthus* mutants, the Trypan blue binding assay was performed as described [24]. In brief, cells growing in liquid CYE medium were collected, adjusted to 5×10^8 cells/ml. 300 μ g protein equivalent of cells was mixed with 1 ml trypan blue working solution (25 μ g/ml trypan blue in cohesion buffer) and incubated in the dark at room temperature for 30 min. The cell suspensions were then pelleted at 16,000 g for 10 min and the absorbance of the supernatants was measured at 585 nm. 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 as reported by Shimkets [25]. Briefly, the exponentially growing cells were harvested, washed and re-suspended to an OD₆₀₀ of 1.0 in MCM buffer (10 mM MOPS, 1 mM CaCl₂, 1 mM MgCl₂). Agglutination or cellular cohesion was determined by measuring absorbance at OD₆₀₀ every 10 min. The experiment was repeated three times.

Co-aggregation Assay

The co-aggregation assay was performed to determine the binding ability of *M. xanthus* strains to *E. coli*. The assay was based on the agglutination assay with some modifications. *M. xanthus* and *E. coli* cells were harvested at the exponential growth phase, and re-suspended to OD₆₀₀ of 1 in MCM buffer. Equal volumes of *M. xanthus* and *E. coli* cell suspensions were thoroughly mixed by vortex for 10 sec and immediately transferred to a plastic disposable cuvette. Co-aggregation was determined spectrophotometrically by measuring absorbance of the supernatant in the cuvette at 600 nm every 10 min. *M. xanthus* cells suspended in the MCM buffer were used as positive control, while *E. coli* cells were used as negative control. Similarly, the binding ability of *M. xanthus* to other bacterial strains, including *K. pneumoniae* IA565, *P. aeruginosa* PA01, and *M. luteus* (Table 1), was also analyzed using the same assay.

Microscopic Evaluation of Co-aggregation between *M. xanthus* and GFP-labeled *E. coli* For a more accurate microscopic assessment of co-aggregation between *M. xanthus* and *E. coli*, GFP-labeled *E. coli* cells were used as co-aggregation partner for *M. xanthus*. The co-aggregation assay was set up as described above. Cell-cell co-aggregation was observed after 30 min using by fluorescence microscopy (Nikon E400 equipped with a mercury lamp and a C17414 filter). GFP-labeled *E. coli* cells suspended in MCM buffer were used as negative control.

Killing Assay in Liquid

M. xanthus and *E. coli* strains were grown to the exponential phase, harvested and re-suspended in the fresh CYE medium to adjust the cell concentration to 5×10^9 cells/ml. *M. xanthus* mutants and *E. coli* were co-cultured (in a 100:1 *M. xanthus*-to-*E. coli* ratio) in liquid CYE medium in a twelve-well plate on a rotary shaker operating at 130 rpm. After a 16 hr incubation at 32 °C, the cell mixture was collected, vortexed thoroughly, serially diluted and spread onto selective LB plates to monitor the viability of *E. coli* cells. Isolated EPS (1 mg/ml) and heat inactivated *M. xanthus* cells (5×10^9 cells/ml) were also used as controls during the killing assay. The kinetics of killing of *E. coli* by *M. xanthus* was further analyzed by monitoring the viability of *E. coli* cells every two hours for twelve hours. Similarly, the

killing ability of *M. xanthus* to other bacterial strains, including *K. pneumoniae* IA565, *P. aeruginosa* PA01, and *M. luteus* (Table 1), was also analyzed using the same assay.

Results

M. xanthus Killed *E. coli* in a Aqueous Environment

Using a model system comprised of *M. xanthus* DK1622 and *E. coli* MG1655 dual species, we aimed to investigate interspecies interaction between *M. xanthus* and *E. coli* cells under aqueous conditions. *M. xanthus* wild type strain DK1622 and *E. coli* strain MG1655 were co-cultured overnight, and the viability of *E. coli* cells was monitored by cfu counting on LB agar plates. As shown in Fig. 1A, the viability of *E. coli* cells suffered five orders of magnitude reduction, from initial 10^7 cells/ml to 10^2 cells/ml after overnight co-cultivation with *M. xanthus* DK1622. Furthermore, the killing kinetics of *M. xanthus* against *E. coli* was analyzed. The most dramatic decline in the number of viable *E. coli* cells occurred in the first two hours, followed by a much slower decrease in the viability of *E. coli* cells with extended incubation time (Fig. 1D). Isolated EPS and heat inactivated *M. xanthus* cells had no *E. coli*-killing effect (data not shown here). The result indicated that, in a aqueous environment *M. xanthus* was still able to kill *E. coli* cells, despite being non-motile and absent of its characteristic solid surface dependent social behaviors [19].

The Observed Killing of *E. coli* by *M. xanthus* Required Direct Cell-cell Contact

To determine if the observed killing of *E. coli* by *M. xanthus* in aqueous condition required direct cell-cell contact between the two species, we employed the two-chamber assay, in which the two different bacterial species shared the same culture medium but were physically separated by a 0.4- μ m membrane. As illustrated in Fig. 1B, when *M. xanthus* cells were inoculated in the lower chamber and *E. coli* cells were incubated in the upper chamber, no significant killing was observed for *E. coli*. Similar growth was monitored for the *E. coli* cells in the upper chamber when they were physically separated from a co-culture of *M. xanthus* and *E. coli* that were incubated in the lower chamber (Fig. 1C). The result suggested that cell-cell contact was essential for the observed killing of *E. coli* by *M. xanthus* under aqueous condition, and that the killing factor(s) involved is unlikely to be freely diffusible.

M. xanthus Cells Co-aggregated with *E. coli* Cells

The ability of *M. xanthus* to bind to *E. coli* cells under aqueous condition was analyzed using the modified co-aggregation assay described above to further demonstrate the contact-dependent interaction of these two species. While *E. coli* cells alone remained dispersed, a drastic reduction in the absorbance at 600 nm was observed for the suspension containing *M. xanthus* and *E. coli* cells (Fig. 2), indicating that the two species formed co-aggregates and precipitated out of the suspension, which resulted in a reduction in the optical density reading. Furthermore, when GFP-labeled *E. coli* was used, the fluorescence microscopy evaluation revealed the formation of co-aggregates containing both *M. xanthus* and *E. coli* cells (Fig. 3).

The EPS, TFP and LPS of *M. xanthus* Are Involved in the Cell-cell Aggregation

Co-aggregation is often mediated by cell surface components. In an initial effort to identify the cell components of *M. xanthus* that are involved in co-aggregation with *E. coli*, mutants that were deficient in the production of cell surface components, including EPS (EPS⁻ mutant SW810, dif⁻ mutant SW504 (*dif* locus is a chemotaxis-like operon, which participates in the regulation of EPS production), TFP (TFP⁻ mutant DK10410), and LPS (LPS⁻ mutant Δ MXAN4619) [26-29], were selected for further investigation. As shown in

Fig. 4, a mixture of *E. coli* cells with *M. xanthus* mutants lacking either EPS or TFP maintained a steady absorbance reading during the 2 hrs incubation period, suggesting they were defective in co-aggregating with *E. coli*. Meanwhile, mixtures containing the LPS mutant also displayed a reduced drop in optical density reading, although not as drastic as observed for the other tested mutant strains. Furthermore, when mixed with GFP-labeled *E. coli*, the *M. xanthus* LPS mutant formed smaller co-aggregates with *E. coli* compared to wild type DK1622; while no obvious aggregates were observed when *E. coli* was mixed with *M. xanthus* EPS⁻ or TFP⁻ mutants (Fig. 3).

EPS⁻, TFP⁻ and LPS⁻ Mutants of *M. xanthus* Were Defective in Killing *E. coli* under Aqueous Condition

Our data showed that killing of *E. coli* by *M. xanthus* was cell-cell contact-dependent and requires the formation of co-aggregation between the two species. To further test if *M. xanthus* mutants defective in co-aggregating with *E. coli* were also deficient in the killing, *E. coli* cells were co-cultivated with these mutants, and their viability was monitored after 16 hrs co-cultivation. Results showed that, compared to the *M. xanthus* wild type strain, all mutants tested displayed various degrees of attenuated *E. coli* killing (Fig. 5A). Interestingly, the killing efficiency of each mutant towards *E. coli* was correlated with its EPS production. Mutants with less EPS displayed more drastic reduction in their ability to kill *E. coli* (Fig. 5).

M. xanthus Demonstrated Different Binding to Different Bacterial Species

Our data suggested an important role of *M. xanthus* EPS in co-aggregating with *E. coli* and mediating the observed contact-dependent killing. To test if EPS-mediated co-aggregation also occurs between *M. xanthus* and other bacterial species, we analyzed the binding ability of *M. xanthus* DK1622 to a panel of different bacterial species, including *K. pneumoniae*, *P. aeruginosa* and *M. luteus*. As shown in Fig. 6A, among all the tested strains, *M. xanthus* DK1622 was able to co-aggregate with *E. coli* and *M. luteus*, while it was unable to co-aggregate with *K. pneumoniae* and *P. aeruginosa*. The more accurate microscopic assessment was employed to demonstrate co-aggregation between *M. xanthus* and *M. luteus*, as *M. luteus* exhibited auto-aggregation in the aqueous condition we tested (Fig. 6B). Our results illustrated the differential co-aggregation ability between *M. xanthus* and other microbial species. More interestingly, *M. xanthus* DK1622 was not able to kill *K. pneumoniae* and *P. aeruginosa* (Fig. 7), the two bacterial species to which *M. xanthus* showed no significant binding under aqueous condition (Fig. 6A).

Discussion

As a model soil bacterium, the surface dependent social behaviors of *Myxococcus*, including its predatory activity against other bacteria, such as *E. coli*, have been extensively investigated [8]. However, little study has been done to investigate the behaviors of *Myxococcus* sp. isolated from aqueous environments. In this study, we revealed for the first time that in an aqueous environment killing of *E. coli* by *M. xanthus* is cell-cell contact-dependent and requires the formation of co-aggregates between the two species (Fig. 1 and Fig. 2). Microbes have evolved many sophisticated strategies to interact and compete with other bacteria to ensure their successful survival in natural environments. Many bacteria can sense the presence of target cells and initiate a series of events in a cell-cell contact dependent manner, which eventually leads to the inhibition or killing of the competing bacteria. Often, the inhibition effect exerted by bacteria can be achieved by physically interacting with target cells and delivering specific effectors via one of the four main classes of bacterial cell surface structures, including type III, IV, V or VI secretion systems [9]. Recently, a novel contact-dependent growth inhibition (CDI) mechanism was revealed, in

which *E. coli* strain EC93 can inhibit the growth of other closely related *E. coli* strains via a two-partner secretion family, including two novel gene products CdiA and CdiB [22]. The contact dependent killing has also been documented in the microbial community-based inhibition of bacteria of foreign origin. He *et al* demonstrated that, an in vitro oral microbial community was able to sense the presence of invading *E. coli* of gut origin in a cell-cell contact dependent manner, and responded by producing hydrogen to kill *E. coli* and prevent its colonization [23].

In our study, the observed killing of *E. coli* by *M. xanthus* under aqueous conditions required formation of co-aggregates. Unlike aforementioned contact dependent inhibition, it appeared that *E. coli* cells were surrounded by *M. xanthus* and were exclusively in the middle in the aggregates (Fig. 3), which may facilitate the killing of *E. coli* by *M. xanthus*. Although we cannot rule out the involvement of other surface structures, such as TFP and LPS in the physical interaction with *E. coli* (Fig. 3 and Fig. 4), our data strongly suggested that EPS was likely the major cell surface component responsible for mediating the formation of co-aggregates between *M. xanthus* and *E. coli*, since the ability of *M. xanthus* to bind to *E. coli* was positively correlated with their EPS production (Fig. 5) and both TFP and *dif* chemotaxis system were reported to participate in the regulation of EPS production [17]. Furthermore, formation of co-aggregates was essential for the killing of *E. coli* by *M. xanthus*, and *M. xanthus* strains defect in co-aggregating with *E. coli* cells were unable to kill *E. coli* in a aqueous environment (Fig. 4 and Fig. 5A). Based on these findings, we propose that the EPS of *M. xanthus* plays an essential role in mediating cell-cell contact dependent *E. coli* killing by *M. xanthus* in a aqueous environment. EPS is a major cell surface component of *M. xanthus* [30] and involved in a variety of cellular functions. EPS is not only required for S-motility and fruiting body formation of *Myxococcus* on solid surface [28], but has also been implicated in its vegetative growth, stress survival and stationary phase recovery in aquatic condition [31]. Furthermore, EPS has been reported to be required for multicellular clumping of *Myxococcus* in aqueous condition [30]. In addition to the involvement of EPS in intraspecies interaction between *M. xanthus* [32, 33], our results further demonstrated its roles in mediating interspecies interaction in aqueous condition.

M. xanthus produces a variety of bacteriolytic/proteolytic enzymes and secondary metabolites [8, 34], which have been suspected to play important roles in killing and competing with other bacteria to enhance their survival and growth at the expense of the target cells. A critical population density was required for effectively hydrolyzing insoluble macromolecules released by target cells [35]. Thus, co-aggregating with *E. coli* under aqueous condition not only allowed for maximum physical interspecies interaction, but also ensured the optimal concentration of myxobacterial bacteriolytic/proteolytic enzymes and secondary metabolites for sufficient killing and feeding of *E. coli* cells. Our data was corroborated by the observation that many newly isolated *Myxococcus* strains from soil failed to grow as dispersed cells in normal liquid broth culture, but formed thin films or clumps instead of living as free cells [17], further suggesting that EPS mediated, contact-dependent killing of prey cells could facilitate the survival of *M. xanthus* in aquatic environments.

It is worth noting that myxobacteria only co-aggregated with certain bacterial species and for those that failed to co-aggregate with *M. xanthus*, they could escape the killing (Fig. 6 and Fig. 7). This result suggested the specific nature of the co-aggregation mediated killing of *E. coli* by *M. xanthus*, which might also require the participation of specific components on the *E. coli* cell surface. Meanwhile, the killing factor(s) involved remained to be determined. Although the myxobacterial antibiotic TA has been reported to be important in killing *E. coli* during predation on solid surfaces [7], TA mutants did not show drastically attenuated killing towards *E. coli* under aqueous condition (data not shown), which

suggested that other killing factors, such as bacteriolytic/proteolytic enzymes were likely involved. Studies are underway to further identify cellular components of *E. coli* involved in co-aggregation as well as the exact killing factor(s) produced by *M. xanthus* during co-aggregation mediated killing under aqueous environment.

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Fig. 1.

Survival of *E. coli* under different conditions A: Wild-type *E. coli* MG1655 was co-cultivated with *M. xanthus* DK1622 in a 1:100 ratio. B: Two-chamber assay in which *E. coli* was inoculated into the upper chamber and physically separated from *M. xanthus* cells in the lower chamber. C: Two-chamber assay in which *E. coli* was added to the upper chamber, while *M. xanthus* cells and *E. coli* (in a 100:1 ratio) was inoculated into the lower chamber. Viability of *E. coli* was monitored after 16 hrs incubation in all conditions. Dark grey bars represent the initial inoculate cell numbers of *E. coli* and grey bars represent viable count of *E. coli* after 16 hrs. The viability measurement of group C is the *E. coli* in the upper chamber. D: Killing kinetics of *M. xanthus* against *E. coli* cells. The viability of *E. coli* cells was monitored every two hours for twelve hours. The experiments were repeated three times. Star indicates significant difference between the two values ($P < 0.05$).

Fig. 2.

Co-aggregation analysis of *M. xanthus* and *E. coli* in MCM buffer A: *M. xanthus* and *E. coli* were analyzed by measuring relative absorbance at 600 nm every ten minutes. Relative absorbance at 600 nm was calculated against the initial OD reading every ten minutes. B: Observed co-aggregation between *M. xanthus* and *E. coli* in static liquid suspensions.

Fig. 3.

Direct observation of the co-aggregation between *M. xanthus* mutants (including dif mutant SW504, EPS mutant SW810, TFP mutant DK10410, and LPS mutant Δ MXAN4619) and GFP-labeled *E. coli* using the fluorescence microscope (Nikon E400 equipped with a mercury lamp and a C17414 filter). The upper panel is micrographs without fluorescence, and the lower panel is fluorescent micrographs. The scale bar equals to 50 μ m.

Fig. 4. Co-aggregation analysis of *M. xanthus* mutants (including *dif* mutant SW504, EPS mutant SW810, TFP mutant DK10410, and LPS mutant $\Delta MXAN4619$) and *E. coli* in MCM buffer. Relative absorbance at 600 nm was calculated against the initial OD reading every ten minutes.

Fig. 5.

EPS production and *E. coli* killing ability of *M. xanthus dif* (SW504), EPS (SW810), TFP (DK10410), and LPS ($\Delta MXAN4619$) mutants in aqueous condition A: Survival of *E. coli* after co-cultivation with *M. xanthus* mutants for 16 hrs. Star indicates significant difference between the two values ($P < 0.05$). B: Relative EPS production of *M. xanthus* mutants determined by Trypan blue assay.

A

M. xanthus + *E. coli* *E. coli* *M. xanthus* + *M. luteus* *M. luteus* *M. xanthus* + *K. p* *K. p* *M. xanthus* + *P. a* *P. a*

B

M. luteus

M. xanthus + *M. luteus*

Fig. 6.

Co-aggregation analysis of different bacteria and *M. xanthus* A: Observed co-aggregation between *M. xanthus* and *E. coli*, *M. luteus*, *K. pneumoniae* (*K. p*) or *P. aeruginosa* (*P. a*) in static liquid suspensions. B: Direct observation of the co-aggregation between *M. xanthus* and *M. luteus* using the microscope (Nikon E400). The scale bar equals to 50 μ m.

Fig. 7.

Survival of *E. coli*, *M. luteus*, *K. pneumoniae* and *P. aeruginosa* after overnight co-cultivation with *M. xanthus* DK1622. Dark grey bars represent the initial inoculate cell numbers and grey bars represent viable count of *E. coli*, *M. luteus*, *K. pneumoniae* and *P. aeruginosa* after overnight co-cultivation with *M. xanthus* DK1622. The experiments were repeated three times. Star indicates significant difference between the two values ($P < 0.05$).

Table 1
Bacterial strains used in this study

Designation	Genotype or description	Reference
<i>Myxococcus xanthus</i> DK1622	Wild type strain	[26]
SW504	DK1622, $\Delta difA$	[27]
DK10410	DK1622, $\Delta pilA$	[36]
SW810	DK1622, $\Delta epsA$	[28]
$\Delta MXAN4619$	DK1622, $\Delta MXAN4619$	This study
<i>Escherichia coli</i> MG1655	Wild type strain	NBRP, National Institute of Genetics Japan
<i>Klebsiella pneumoniae</i> IA565	Clinical isolate	[20]
<i>Pseudomonas aeruginosa</i> PA01	Wild type strain	Laboratory strain
<i>Micrococcus luteus</i>	Collection from ATCC	ATCC272