A two-component regulatory system modulates twitching motility in *Dichelobacter nodosus*

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HIGHLIGHTS

- A two-component regulatory system, TwmRS, with similarity to chemosensory systems was identified in *Dichelobacter nodosus*
- Evidence was obtained that this system modulates twitching motility, with a *twmR* mutant showing decreased twitching motility
- Video microscopy indicated that the twmRS system controls the direction of movement by twitching motility
- The TwmRS system may play a role in disease, since Type IV fimbriae-mediated twitching motility is essential for virulence in *D. nodosus*

ABSTRACT

Dichelobacter nodosus is the essential causative agent of footrot in sheep and type IV fimbriae-mediated twitching motility has been shown to be essential for virulence. We have identified a two-component signal transduction system (TwmSR) that shows similarity to chemosensory systems from other bacteria. Insertional inactivation of the gene encoding the response regulator, TwmR, led to a twitching motility defect, with the mutant having a reduced rate of twitching motility when compared to the wild-type and a mutant complemented with the wild-type *twmR* gene. The reduced rate of twitching motility was not a consequence of a reduced growth rate or decreased production of surface located fimbriae, but video microscopy indicated that it appeared to result from an overall loss of twitching directionality. These results suggest that a chemotactic response to environmental factors may play an important role in the *D. nodosus*-mediated disease process.

1. **Introduction**

Dichelobacter nodosus is a Gram-negative aerotolerant anaerobe that is the essential causative agent of ovine footrot, a debilitating disease of the feet of sheep. Footrot is characterised by separation of the keratinous hoof from the underlying tissue, which leads to lameness and loss of body condition and is therefore of major economic importance to the wool and sheep meat industries (Stewart, 1989). The major virulence factors of *D. nodosus* are the type IV fimbriae (Kennan et al., 2001) and extracellular serine proteases (Kennan et al., 2010; Kortt et al., 1994; Kortt et al., 1993).

Type IV fimbriae are produced by many Gram-negative bacteria, have a polar location and are responsible for a flagellum-independent form of motility known as twitching motility (Mattick, 2002). Twitching motility is essential for virulence in *D. nodosus*, since disruption of the *pilT* gene results in mutants that still produce fimbriae, are unable to undergo twitching motility and are avirulent in a sheep virulence model (Han et al., 2008).

Two-component regulatory systems are signal transduction systems that allow bacteria to sense and respond to changes in their environment (Stock et al., 2000). Typical systems consist of a sensor histidine kinase, which senses an extracellular stimulus that leads to autophosphorylation and the subsequent phosphorylation of its cognate response regulator (Casino et al., 2010). The response regulator often has a C-terminal DNA-binding domain, which is activated by phosphorylation of the N-terminal receiver domain and subsequently acts as a repressor or activator of gene expression (Wadhams and Armitage, 2004). However, some response regulators, such as CheY from the *Escherichia coli* chemotaxis system, are single domain proteins that upon phosphorylation bind directly to specific target proteins and modulate their function.

Chemotaxis is the movement towards or away from chemicals that are beneficial or toxic, respectively, and the signalling pathways involved are well studied in *E. coli* and *Salmonella enterica* serovar Typhimurium (Sourjik and Wingreen, 2012; Wadhams and Armitage, 2004). The *E. coli* chemotaxis system consists of six essential proteins CheA, CheW, CheY, CheZ, CheR, and CheB and associated chemoreceptors (Baker et al., 2006a). The chemoreceptors, or methyl accepting chemotaxis

proteins (MCPs), detect the external signal and facilitate the phosphorylation of the sensor histidine kinase, CheA. The phosphoryl group is then transferred to the response regulator CheY, which after phosphorylation binds to the FliM protein, a component of the flagellar motor, and causes a reversal in the direction of the motor (Wadhams and Armitage, 2004). CheW links the MCPs and CheA, CheZ dephosphorylates CheY, and CheB and CheR modify the chemoreceptors to provide a memory response (Baker et al., 2006a). CheA, CheW and CheY are highly conserved in all bacterial chemotaxis systems (Baker et al., 2006b; Sourjik and Wingreen, 2012).

Sequencing of the genome of the virulent *D. nodosus* strain VCS1703A identified five two-component regulatory systems (Myers et al., 2007). These systems included PilSR, which is required for the expression of the type IV fimbriae subunit gene, *fimA* (Parker et al., 2006), and a system that has similarity to a CheAY-like chemosensory system, which we now designate as TwmRS (Myers et al., 2007). Subsequent studies have shown that the genes encoding these regulatory systems are present in all 103 *D. nodosus* strains that have been sequenced (Kennan et al., 2014). Here we report the further characterisation of the TwmRS system and show that it is involved in the modulation of twitching motility.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. *D. nodosus* strains were routinely grown in an anaerobic chamber (Coy Laboratory Products Inc.) in an atmosphere of 10% (v/v) H_2, 10% (v/v) CO_2, and 80% (v/v) N_2 on Eugon (Difco) yeast extract (EYE) agar with 5% (v/v) defibrinated horse blood. When required erythromycin (1µg/ml) or kanamycin (10 µg/ml) were added for the selection of transformants. For the preparation of competent cells and culture supernatants, strains were grown in trypticase arginine serine (TAS) broth (Skerman, 1975). All *E. coli* strains were grown in

2YT media (Maniatis et al., 1982), with ampicillin (100 μg/ml) and kanamycin (20 μg/ml) added as required.

2.2. Construction of twmR mutant and complementation

All molecular techniques were performed using standard procedures. The *twmR* suicide plasmid, pJIR2566, was constructed by inserting a 1.5 kb PCR product containing *twmR* and part of *twmS* at the *Eco*RI and *Kpn*I sites of pWSK29 (Wang and Kushner, 1991). The *ermB* gene was then inserted into the *Bst*EII site located near the centre of the cloned fragment. The resultant suicide vector was then introduced into *D. nodosus* strain VCS1703A by natural transformation (Kennan et al., 2001), selecting for mutants on medium containing erythromycin. The *twmR* complementation plasmid pJIR3404 was constructed by inserting the full-length *twmR* gene, upstream of a kanamycin resistance gene, between the *D. nodosus rrnA* promoter and terminator. This plasmid was then introduced into the *twmR* mutant JIR3931 by natural transformation, selecting for kanamycin resistance. The *twmR gene* and kanamycin resistance genes were inserted onto the chromosome at one of the three *rrnA* operons.

2.3. Phenotypic assays

Twitching motility assays and quantitative protease assays were performed as described previously (Kennan et al., 2001). Immunoblotting was performed as before (Han et al., 2007).

2.4. Microscopy

D. nodosus cultures were incubated for four days on TAS agar at 37°C in an anaerobic chamber. Isolated single colonies and twitching motility zones were imaged using an Olympus 1X71 inverted microscope fitted with a CC12 colour camera and AnalySIS LS software. For video microscopy, *D. nodosus* cultures were stab inoculated into 3 mm thick TAS agar in 3.5 cm petri dishes and incubated

anaerobically at 37°C for 4 days. Petri dishes then were sealed in oxygen impermeable bags (Difco) and removed from the anaerobic chamber. The petri dish (in the bag) was then placed on the heated microscope stage (37°C). An image of the edge of the twitching zone was taken every 30 s for 100 min using an FViewII monochrome camera. The distance that the edge of the twitching zone had moved in 100 min was used to calculate a twitching motility rate.

2.5. Bacterial two-hybrid assay

A bacterial two-hybrid system (Karimova et al., 2000) was used to identify protein-protein interactions. Potential interacting proteins were cloned separately into the vectors pKT25, pUT18 and pUT18C, and the resultant constructs were used to co-transform *E. coli* strain BTH101. Controls consisted of a vector expressing a fusion protein and a corresponding empty vector. Transformants of interest were tested in a quantitative ß-galactosidase assay as described previously (Teng et al., 2008).

2.6. Pull-down assay

The *twmR* gene was tagged with a His epitope by cloning the *twmR* PCR product into the vector pCR8/GW/TOPO (Invitrogen) and then into pDEST17 (Invitrogen), after confirmation of the sequence. *E. coli* BL21cells containing this construct were grown to exponential phase before treatment with 1mM IPTG for 4 h at 37°C for the expression of His-tagged TwmR. Cells were collected by centrifugation and resuspended in binding/wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 0.1% Tween 20) and then the cells were lysed by sonication or in a Precellys 24 tissue homogenizer (Bertin Technologies). The cell debris was deposited by brief centrifugation and the supernatant mixed with Dynabeads® His tag isolation and pull-down (Invitrogen) as per the manufacturer's instructions. After binding of the His-tagged TwmR and washing, a whole cell lysate of *D. nodosus* cells in pull-down buffer (3.25M sodium phosphate, pH 7.4, 70 mM sodium chloride, 0.01% Tween 20) was added to the beads and treated as per the manufacturer's instructions. After removal of unbound proteins, beads were

washed in binding/wash buffer four times and the bound proteins eluted in His-elution buffer (300 mM imidazole, 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 0.01% Tween 20). Eluted proteins were analysed by SDS-PAGE and mass spectrometry.

2.7. Bioinformatics

The relationship between TwmS or TwmR to other bacterial proteins was determined using standard procedures (Altschul et al., 1990; Altschul et al., 1997). Domain analysis was completed using the Simple Molecular Architecture Tool (SMART) (Schultz et al., 1998), while PSORTb (Gardy et al., 2005) and TMpred (Hofmann, 1993) were used to predict cellular location and transmembrane regions respectively. TwmR was also analysed using STRING (Franceschini et al., 2013) to identify possible interacting proteins.

3. Results

3.1. TwmSR comprises a chemosensory two-component system

Sequencing of the genome of *D. nodosus* strain VCS1703A identified a two component regulatory system (DNO_0868 and DNO_0869) with similarity to CheA/CheY two component systems (Myers et al., 2007). We have designated these genes as *twmR* and *twmS*, respectively. TwmS is a 736 aa protein that has similarity to CheA proteins and is predicted to have two N-terminal transmembrane domains and to contain C-terminal histidine phosphotransfer (HPT) and histidine kinase-like ATPase (HATPase_c) domains. Proteins most similar to TwmS are a putative CheA protein (37% identity) from *Psychrobacter cryohalolentis K5* (Genbank Accession No. YP 579652) and *Psychrobacter* sp. PAMC21119 (36% identity) (WP010201764.1), a CheA protein from *Simonsiella muelleri* ATCC29453 (WP002641487.1), a putative CheA protein from *Kingella oralis* ATCC51147 (35% identity) (WP003798491.1) and *Kingella kingae* strains PYKK081 (WP0037889112.1) and ATCC23330

(WP003787205.1). This similarity extended across the full length of the TwmS protein. TwmR is predicted to be a 13 kDa, 122 aa protein with a predicted receiver (REC) domain showing significant sequence similarity to response regulator receiver domain proteins from *Kingella oralis* ATCC511471 (65% identity) (WP003798489.1), a response regulator protein from *Psychrobacter sp.*PAMC21119 (62% identity) (WP010201763.1) and a CheY like protein from *Neisseria* sp. oral taxon 020 StrF0370 (62% identity) (WP009425881).

3. 2. A twmR *mutant displays a twitching motility defect*

A chromosomal mutant of *twmR* was constructed by allelic replacement, using the suicide vector pJIR2566, which contained a copy of *twmR* insertionally inactivated with the erythromycin resistance gene *ermB.* The mutant was confirmed by PCR and Southern hybridisation (data not shown). The *twmR* mutant was complemented by insertion of a wild-type copy of *twmR* onto the chromosome by homologous recombination using plasmid pJIR3404, which inserted the *twmR* gene into one of the three *rrn* operons. The genotype of the complemented strain also was confirmed by PCR and Southern hybridisation (data not shown).

When a standard twitching motility assay was performed (Kennan et al., 2001) on the wild-type, mutant and complemented strains, the *twmR* mutant showed a marked reduction in the size of its twitching zone when compared to the wild type (Fig. 1A). This effect was reversed by complementation with the wild-type gene (Fig. 1A). Time-lapse video microscopy then was used to determine the rate of twitching motility of each of the strains, and also to observe any differences in cell movement, with each strain observed on three separate occasions. The average twitching motility rate of the *twmR* mutant was 0.58 μm/min, which was significantly different to both the wild-type (1.8 μm/min) and the complemented strain (1.0 μm/min) ($p<0.05$) (Fig. 2). Careful examination of the twitching motility movies of each strain showed that the wild-type cells (see Supplementary Movie 1) moved towards the outer edge of the twitching zone, with the majority of cells appearing to move in that general direction. The overall movement of the mutant, however, was less directional with cells appearing to move more in

circles rather than towards the twitching zone edge (Supplementary Movie 2). Complementation restored the movement of cells to the more directional movement of the wild type (Supplementary Movie 3). Microscopic examination of single colonies showed a difference in colony morphology between the wild-type and mutant, with the surface of the mutant colony appearing rougher and to contain mounds of cells, while the colonies of both the wild-type and complemented strain appeared more even (Fig. 1B). The outer "fringe" of the colonies represented areas with the most active twitching motility. The smooth centre of the wild-type colonies was likely to be a consequence of cells having stopped or slowed twitching motility and switched to a more sedentary growth mode. The roughness of the *twmR* mutant colonies would be consistent with continued twitching motility within the outer edges and central parts of the colonies.

3.3. Other fimbriae-associated functions were not affected by the twmR *mutation*

To further confirm that the twitching motility defect that was observed was due to the disruption of the *twmR* gene and not to other changes we examined the growth rate, fimbriae production and protease secretion of the wild-type, mutant and complemented strains. Growth curves were performed over 48 h and the results showed that there was no significant difference in the growth rates of the wild-type, mutant and complemented strains (data not shown). Immunoblotting of surface fimbriae with fimbriae-specific antiserum showed that each of these strains produced an equivalent amount of surface fimbriae (Fig.3A), indicating that the reduced twitching motility rate was not due to a reduction in the amount of fimbriae produced by the mutant. Since previous studies have also shown that the fimbrial subunit (FimA) (Kennan et al., 2001) and twitching motility (Han et al., 2008) are required for the secretion of wild-type levels of extracellular proteases, we therefore determined the extracellular protease activity of the isogenic strains; there was no significant difference in the level of protease secretion (Fig. 3B).

3.4. Protein-protein interactions involving TwmR and TwmS

In an attempt to understand the mode of action of TwmR we used a bacterial two-hybrid system (Karimova et al., 2000) to look for interactions between TwmR and TwmS and between TwmR and the fimbrial biogenesis proteins FimN, FimO, PilT and PilU, all of which are involved in the assembly or retraction of *D. nodosus* fimbriae. Since TwmR, like CheY, did not have a DNA-binding effector domain it was postulated that it modulates the direction of twitching motility by binding directly to a component of the fimbrial biogenesis system. Using this bacterial two-hybrid, β-galactosidase-based, reporter system, interactions were detected between TwmR and TwmS and between TwmS and TwmS (Fig. 4), with β-galactosidase activity being similar to that of the positive control and significantly different to the negative control ($p<0.05$). However, no significant interactions were detected between TwmR and the fimbrial biogenesis proteins using this system (data not shown).

In an attempt to identify other candidate proteins that may interact with TwmR we analysed TwmR using STRING (Franceschini et al., 2013). This program identified PilH, PilJ, PhoR, DNO_0867 (a CheC like protein) and DNO_0866 (a putative nitroreductase), as well as TwmS, as possible interacting proteins. Each of these proteins, except for PhoR, which could not be cloned, was cloned into the bacterial two-hybrid system and tested for interactions with TwmR and TwmS. Interactions were only detected between TwmS and PilJ, however, both of these proteins contain transmembrane domains, and it was thought that interactions may have been detected because of co-localisation to the membrane. When the regions encoding the putative transmembrane domains of both of these proteins were removed and the resultant genes cloned into the bacterial two-hybrid system no interactions were detected (Fig. 4B).

Another method by which protein-protein interactions can be detected is through pulldown assays. To this end attempts were made to construct a His-tagged TwmR derivative in *D. nodosus* so that the protein could be purified, effectively in its native form, and interacting proteins detected by co-purification. An initial construct was made encoding a C-terminal hexahistidine tag (TwmR-His_6), but unfortunately this protein was inactive; the resultant complemented *D. nodosus* strain had the same twitching motility phenotype as the *twmR* mutant. When a second construct was made with an N-terminal

tag (His_6-TwmR), the resultant complemented twitching motility profile was normal, but no His-tagged protein could be detected in a Western blot. A His-tagged TwmR protein subsequently was purified from an *E.coli* expression strain and used in pull-down assays with *D. nodosus* whole cell lysates, but no interacting proteins were identified.

4. Discussion

Twitching motility is a flagella-independent form of bacterial motility mediated by type IV fimbriae (Burrows, 2012) and we have previously shown that type IV fimbriae-mediated twitching motility is essential for the virulence of *D. nodosus* in sheep (Han et al., 2008). It is assumed that this form of motility enables *D. nodosus* cells to move deeper into the developing lesion, thereby finding a more anaerobic environment so that they can proliferate and secrete extracellular proteases, which then cause tissue damage. The results presented in this paper show that twitching motility in *D. nodosus* also is modulated by a two-component regulatory system that shows similarity to bacterial chemosensory systems. This system consists of a sensor kinase (TwmS), the C-terminal domain of which has a HPT domain and a HATPase_c domain, which is similar to domains present in CheA-like sensor histidine kinases. The cognate response regulator TwmR contains a receiver domain that shows similarity to CheY-like proteins, which do not have a C-terminal DNA binding domain.

Insertional inactivation of the *twmR* gene resulted in a mutant with a twitching motility defect. This defect was restored in the complemented strain, although not quite to wild-type levels, presumably because complementation was at a different site in the genome. The mutation appeared to only affect the twitching motility process, not fimbrial biogenesis, since the mutant still produced equivalent amounts of fimbriae to the wild-type and had normal levels of extracellular proteases. Since it could be complemented the mutant still was able to undergo fimbriae-dependent natural transformation, which provides further evidence for normal fimbrial function. The major effect of this mutation appeared to be an alteration in the directionality of twitching motility-mediated movement, leading to an overall reduction in the rate of progress in any one direction across an agar surface.

The velocity of cells undergoing twitching motility depends on the rate of fimbrial retraction (Mignot and Kirby, 2008). A recent model for Type IV pilus assembly and retraction (Craig and Li, 2008) suggests that conformational changes in the assembly ATPase (FimN in *D. nodosus*) and the retraction ATPase (PilT in *D. nodosus*, potentially with the involvement of PilU), driven by ATP hydrolysis, mediates pilus assembly and retraction, respectively (Han et al., 2008). For this reason, logical sites for the TwmR interactions that modified twitching motility were FimN, PilT and PilU, all of which are ATPases involved in the assembly and retraction of fimbriae in *D. nodosus* (Han et al., 2008; Han et al., 2007). Another potential target site is the inner membrane platform protein, FimO (Johnston et al., 1998). We were unable to show any interactions between TwmR and any of these proteins using a bacterial two-hybrid system, but this does not mean these interactions do not occur. The interactions may only occur if TwmR is phosphorylated, which may not be the case in this heterologous *E. coli* system, or the interactions may be too transient to be detected. However, interactions were detected between TwmS and TwmS and between TwmS and TwmR indicating that the bacterial two-hybrid system was functional for these proteins under the conditions tested. Similarly, interactions were detected between TwmS and PilJ, but these may have been due to co-localisation of these proteins to the bacterial membrane as no interactions were detected when the potential transmembrane domains of these two proteins were removed. Attempts to identify other proteins that may interact with TwmR using pull-down assays were unsuccessful.

It is also possible that TwmR may exert its effect indirectly, through other proteins which may or may not interact with one or more of the proteins already tested. Another chemosensory system that may exert an effect on twitching motility is encoded on the *D. nodosus* genome (Myers et al., 2007), this Chp system has similarity to a complex chemosensory system that controls twitching motility in *Pseudomonas aeruginosa* (Whitchurch et al., 2004). This system is yet to be investigated in detail in *D. nodosus*, but since it shows many similarities to the *P. aeruginosa* Chp system, and other aspects of fimbrial biogenesis are similar and even interchangeable in these two organisms (Johnston et al., 1995; Johnston et al., 1998), it is possible that the Chp system may also exert some control over twitching motility in *D. nodosus*, either via TwmRS or independently. Interestingly, mutants of *pilH*, which

encodes a CheY homolog component of the *P. aeruginosa* Chp system, show similar twitching defects to *D. nodosus twmR* mutants in that they also appear to move in swirls (Darzins, 1994).

The chemotactic signals that may influence twitching motility in *D. nodosus* have not been identified, however, there are several possibilities including components of the sheep hoof, the amino acids serine and arginine, which have previously been shown to be important for the growth of *D. nodosus* (Rood et al., 2005), or long chain fatty acids, which are chemoattractants to *P. aeruginosa* and *M. xanthus* (Kearns and Shimkets, 1998; Miller et al., 2008). Attempts were made to identify compounds that may influence the direction of twitching in *D. nodosus* by setting up gradients of serine, arginine and hoof powder in agar plates as well as phosphotidylethanolamine (PE) and 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine gradients as described for *P. aeruginosa* (Miller et al., 2008), but these experiments were unsuccessful. The slow rate of growth of *D. nodosus* may have been a factor in these assays with the putative chemical attractant diffusing through the agar too quickly; it generally takes three days to be able to see *D. nodosus* twitching motility zones.

In conclusion, we have shown that twitching motility in *D. nodosus* is modulated by a two-component regulatory system that shows similarity to chemotaxis systems from other bacteria. The TwmSR system controls the rate of twitching motility, with a *twmR* mutant not moving outwards towards the edge of the twitching zone as rapidly as the wild type. Since we know that twitching motility is essential for the virulence of *D. nodosus* in sheep (Han et al., 2008), it is also likely that TwmSR-mediated regulation of twitching motility may also influence the virulence of isolates and subsequent disease progression.

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447 **Figure Legends**

448

449 **Fig. 1.** Differences in twitching motility and colony morphology. A. Twitching motility assay of the
450 isogenic strains. Each strain was stab inoculated into a 1% TAS agar plate and incubated for seven days
451 at 37˚C. Twitching motility zones were then stained with Coomassie Blue. The scale bar represents 1 cm.
452 B. Microscopic analysis of colony morphology. Single colonies that had been growing anaerobically for
453 four days at 37˚C were photographed. The scale bar represents 500µm. Legend: WT (wild-type strain
454 VCS1703A), *twmR* (the twmR mutant JIR3931), *twmR/twmR*$^+$ (the complemented *twmR* mutant
455 JIR3932).

456

457 **Fig. 2.** Twitching motility rates of the isogenic strains. The wild-type (VCS1703A), *twmR* mutant
458 (JIR3931) and *twmR* complemented strain (JIR3932) were grown on 1% TAS agar for 4 days. The graph
459 shows the mean and SEM of three biological replicates for each strain. The twitching motility rate of the
460 *twmR* mutant was significantly different to both the wild-type and complemented strains (p<0.05)
461 Student's *t* test.

462

463 **Fig. 3.** Fimbrial production and protease secretion. A. Western immunoblot of purified cell surface
464 fimbriae from the isogenic strains. Samples were separated by electrophoresis on 12% SDS-PAGE,
465 blotted to nitrocellulose and probed with fimbrial serogroup G specific antiserum. B. Quantitative
466 analysis of protease production. Protease activity in culture supernatants of the isogenic strains was
467 determined using azocasein as substrate. The graph shows the mean and SEM of three biological
468 replicates. Legend: WT (wild-type strain VCS1703A), *twmR* (the *twmR* mutant JIR3931*), twmR/twmR*$^+$
469 (the complemented *twmR* mutant JIR3932).

470

471 **Fig. 4.** Protein-protein interactions identified by bacterial two-hybrid analysis. *E. coli* BTH 101 cells
472 were co-transformed with pKT25 fused to the DNA sequence encoding TwmS, and (A) pUT18 (1) and
473 pUT18c (2) encoding TwmS or TwmR and (B) pUT18 derivatives encoding full-length TwmS or PilJ or

TwmS or PilJ without their predicted transmembrane domains (TwmS(s) and PilJ(s)). Negative controls consisted of BTH101 cells co-transformed with the vector expressing the test protein (pUT18 or pUT18c) and the control plasmid pKT25. Quantitative β-galactosidase assays were performed in triplicate with the mean and SEM shown.

Table 1

Bacterial strains and plasmids

Strain	Characteristics	Source
D. nodosus		
VCS1703A	Virulent serogroup G strain	J. Egerton, University of Sydney. (Kennan et al., 2014)
JIR3931	VCS1703A*twmRΩermB*	Double crossover from pJIR2566. Selected erythromycin resistance.
JIR3932	JIR3931*rrnA*Ω*twmR*$^{+}$	Double crossover from pJIR3404. Selected kanamycin resistance.
E. coli		
DH5α	F^{-}*endA1 hsdR17*($r_k^-m_k$)*thi-1λ*$^{-}$*recA1 gyrA96relA1 rhoA supE44 deoRΦ80d lacZ*ΔM15*Δ(lacZYA argF)*U169	Invitrogen
BTH101	F^{-} *cya-99 araD139 galEIS galK16 rpfL1 hfdV2 mcrB1*	(Karimova et al., 2000)
BL21 Star™ (DE3)	F^{-}*omp*T *hsd*S_B (r_B^-, m_B^-) *galdcmrne*131 (DE3)	Invitrogen
Plasmids		
pBluescriptSK^{+}	Amp^{r}, *lacZ* cloning vector	Stratagene
pWSK29	Amp^{r}, *lacZ* cloning vector	(Wang and Kushner, 1991)
pKT25	Km^{r} cloning vector for bacterial two-hybrid system	(Karimova et al., 2000)
pUT18	Amp^{r} cloning vector for bacterial two-hybrid system	(Karimova et al., 2000)
pUT18C	Amp^{r} cloning vector for bacterial two-hybrid system	(Karimova et al., 2000)
pKT25-zip	Km^{r} positive control for bacterial two-hybrid system	(Karimova et al., 2000)
pUT18C-zip	Amp^{r} positive control for bacterial two-hybrid system	(Karimova et al., 2000)
pJIR2558	pBluescriptSK^{+} *Kpn*1/*Sac*II Ω 1.5 kb fragment containing *twmR*	Recombinant
pJIR2566	pJIR2558 *Bst*EII Ω *ermB*	Recombinant
pJIR2680	pWSK29*Xba*I/*Xho*IΩ*D. nodosus rrnA* promoter Km^{R} *D.nodosus rrnA* terminator	Recombinant
pJIR3404	pJIR2680 *Bam*HI Ω *twmR*	Recombinant
pCR8/GW/TOPO	TOPO TA cloning entry vector	Invitrogen
pDEST17	Gateway® destination vector	Invitrogen
pJIR3908	pCR8/GW/TOPOΩ *twmR*	Recombinant
pJIR3910	pDEST17Ω *twmR*	Recombinant

Figure 1

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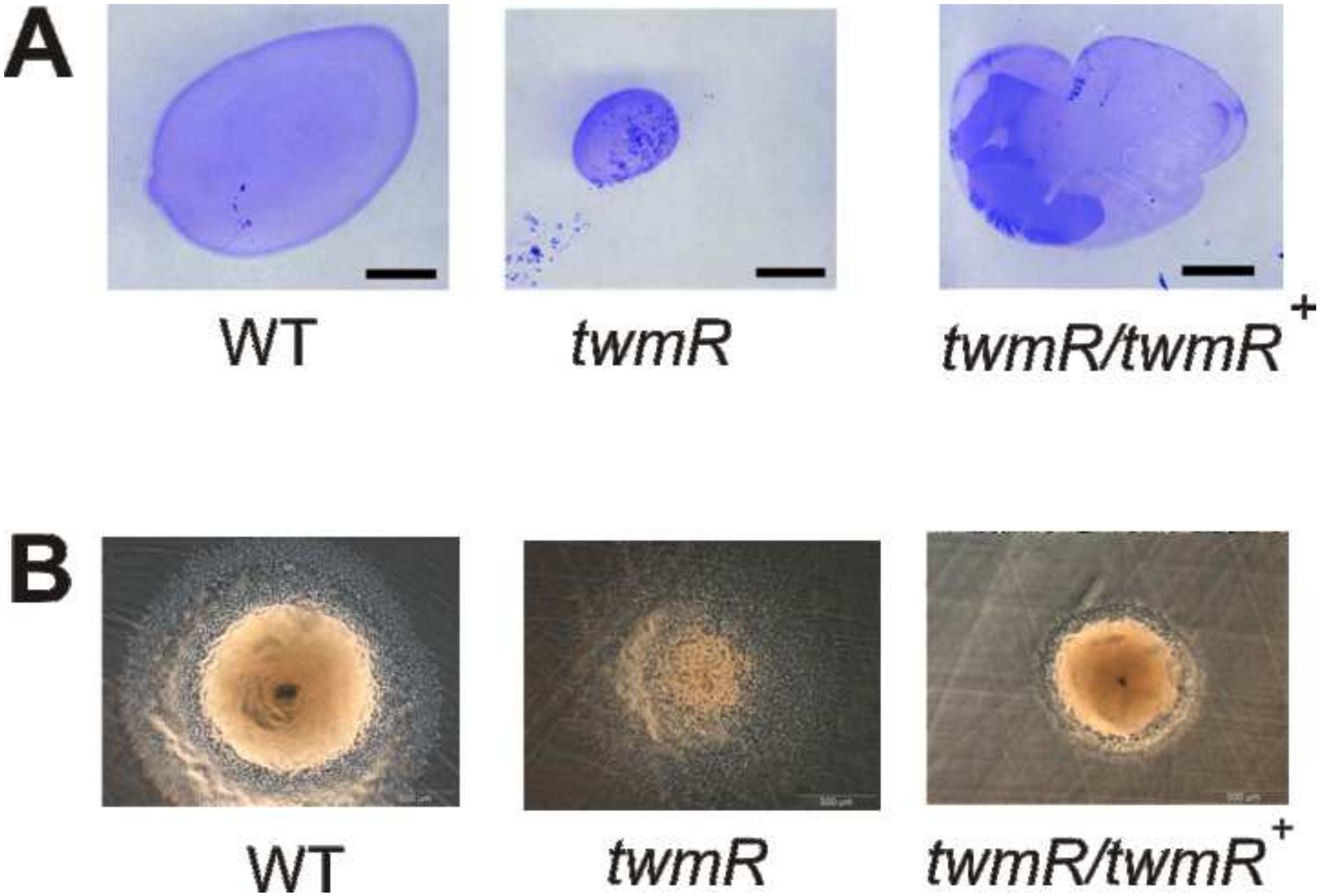

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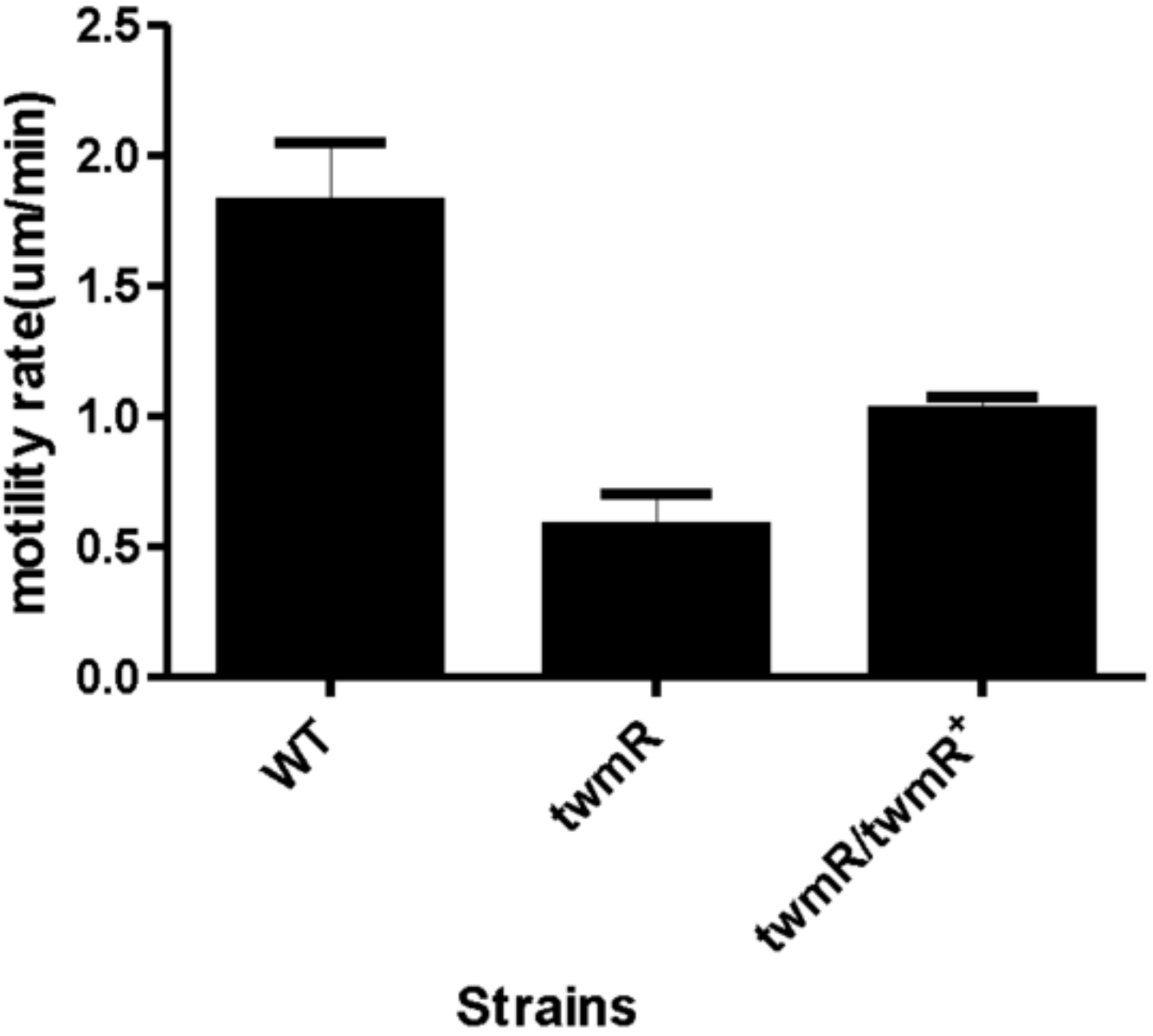

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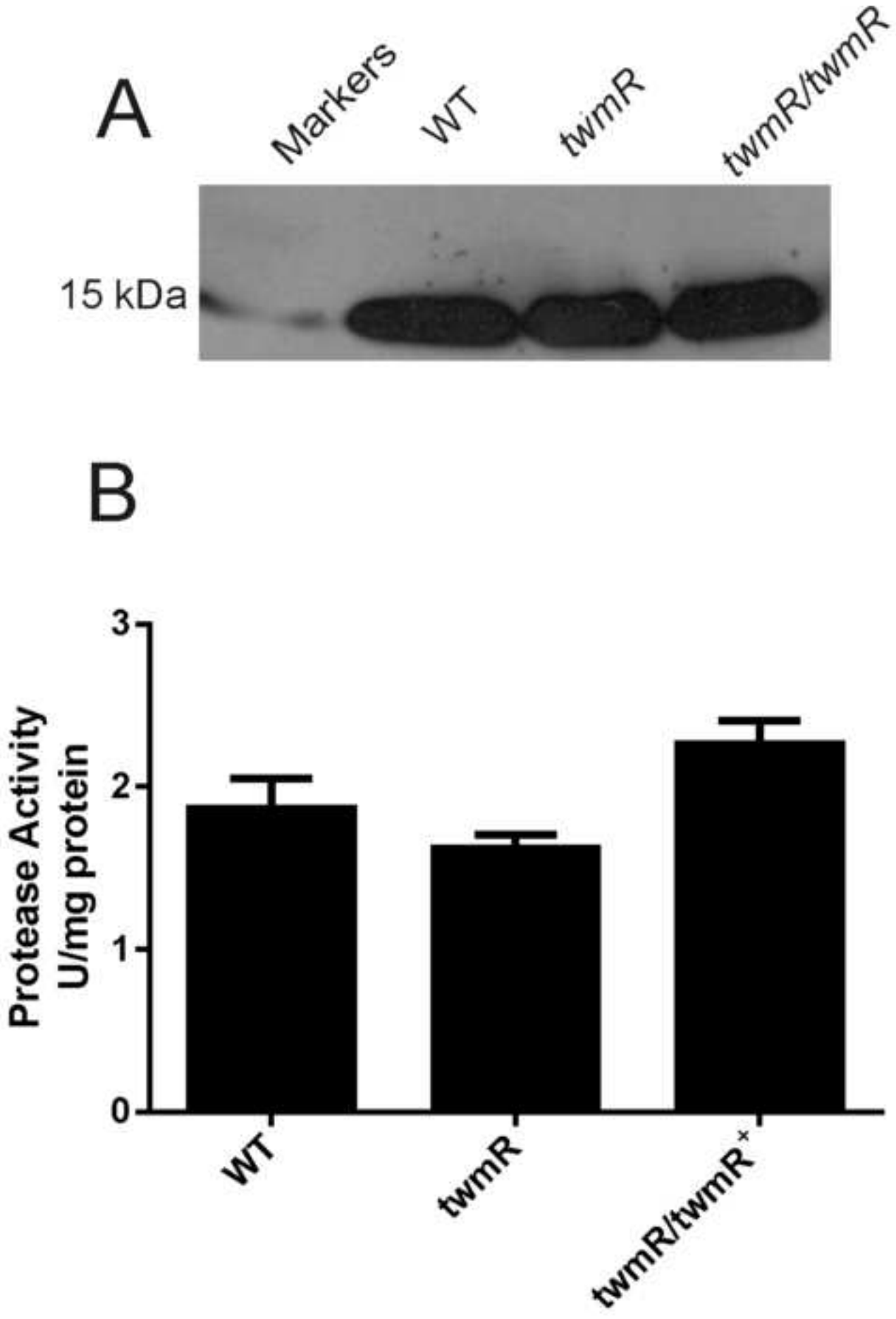

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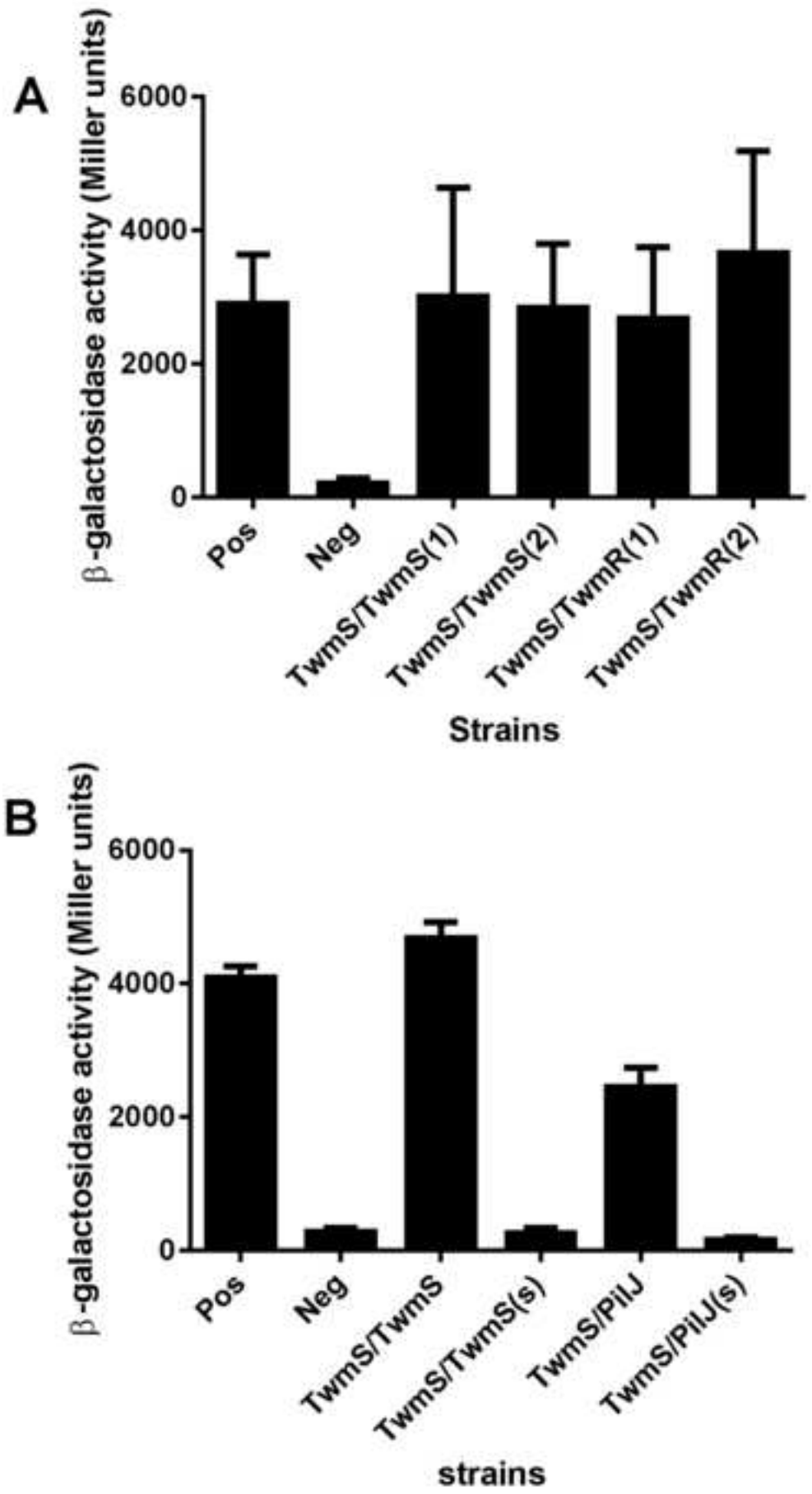

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