ExpR is not required for swarming but promotes sliding in *Sinorhizobium meliloti*.

Joaquina Nogales, Lydia Bernabéu-Roda, Virginia Cuéllar, and María J. Soto#.

Dpto. Microbiología del Suelo y Sistemas Simbióticos. Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda 1, 18008 Granada, Spain.

#Corresponding author. Mailing address: Dpto. Microbiología del Suelo y Sistemas Simbióticos. Estación Experimental del Zaidín. 18008 Granada (Spain).

Phone: +34-958181600. Ext. 150 Fax: +34-958129600

E-mail: mariajose.soto@eez.csic.es

Running title: Role of ExpR in surface motility

Swarming is a mode of translocation dependent on flagellar activity that allows bacteria to move rapidly across surfaces. In several bacteria, swarming is a phenotype regulated by quorum-sensing. It has been reported that the swarming ability of the soil bacterium *Sinorhizobium meliloti* Rm2011 requires a functional ExpR/Sin quorum sensing system. However, our previous published results demonstrate that strains Rm1021 and Rm2011, both known to have a disrupted copy of *expR*, are able to swarm on semisolid minimal medium. In order to clarify these contradictory results, the role played by the LuxR type regulator ExpR has been re-examined. Results obtained in this work revealed that *S. meliloti* can move over semisolid surfaces using at least two different types of motility. One type is flagella-independent surface spreading or sliding which is positively influenced by a functional *expR* gene mainly through the production of exopolysaccharide II (EPS II). To a lesser extent, EPS II-deficient strains can also slide on surfaces by a mechanism that is at least dependent on the siderophore rhizobactin 1021. The second type of surface translocation shown by *S. meliloti* is swarming which is greatly dependent on flagella and rhizobactin 1021 but does not require ExpR. We have extended our study to demonstrate that the production of normal amounts of succinoglycan (EPS I) does not play a relevant role in surface translocation but its overproduction facilitates both swarming and sliding motilities.

1 Bacteria can move using different types of translocation. Swimming is a flagella-
2 driven motility that takes place in liquid environments. Bacterial translocation over
3 surfaces can occur by twitching, gliding, sliding and swarming (18,19). Twitching is a
4 slow cell movement on surfaces that is mediated by the extension and retraction of type
5 IV pili. Gliding, a surface translocation extensively studied in myxobacteria, does not
6 require flagella or pili but involves focal-adhesion complexes, cell surface-associated
7 complexes that anchors the bacterium to a substrate and might act as a motor. Sliding or
8 spreading by expansion has been described as a passive surface translocation that is
9 powered by the outward pressure of bacterial growth and facilitated by compounds that
10 reduce friction between cells and surfaces. Swarming is a mode of surface translocation
11 dependent on rotating flagella characterized by the rapid and co-ordinated movement of
12 multicellular groups of bacteria. It is considered the fastest known type of bacterial
13 motility on surfaces, with speeds of translocation very similar to the swimmer's speeds
14 (up to 40 μm/s) (18). This allows swarmer cells to rapidly colonize different
15 environments. An additional and distinguishing feature of swarming is that it can
16 involve a complex process of morphological and physiological differentiation. Cells
17 usually (but not always) become hyperflagellated and elongated, and substantial
18 alterations in metabolic pathways and gene expression have been observed (24,33,46).
19 This process is known to be triggered upon integration of several chemical and physical
20 signals (12,23,45). Swarming has been described as a quorum sensing-regulated
21 phenotype in several bacteria (8). Quorum sensing systems have been reported to be
22 involved in the production of biosurfactants that act as wetting agents which reduce the
23 surface tension during surface migration, and in swarmer cell differentiation.

24 Swarming motility is not well characterized in the soil bacteria collectively known as
25 rhizobia that are able to establish nitrogen-fixing symbiosis with legume plants. To date,

1 within rhizobia this surface motility has been described in *Sinorhizobium meliloti*,
2 *Rhizobium etli* and *R. leguminosarum* biovar viciae (7,42,44). *R. etli* has been
3 demonstrated to have a quorum sensing-regulated swarming motility: mutations
4 affecting the *cinIR* quorum sensing system abolish surface translocation in this
5 bacterium. Moreover, it has been shown that *N*-acyl-homoserine lactones (AHLs)
6 carrying a long-chain fatty acid moiety have a dual role in swarming of *R. etli*: as
7 quorum sensing signals and as biosurfactants which promote surface translocation (7).

8 *S. meliloti* possesses the ExpR/Sin quorum sensing system which is composed of two
9 transcriptional regulators, ExpR and SinR, and the autoinducer synthase SinI which is
10 responsible for the synthesis of several AHLs (26). The *sin* AHLs together with ExpR
11 control the expression of a large number of genes involved in several free-living and
12 symbiotic cell functions such as the production of the exopolysaccharides (EPS)
13 succinoglycan (EPS I) and galactoglucan (EPS II), or motility (13,16,21,22). In *S.*
14 *meliloti*, the expression of motility genes is down-regulated at high population densities.
15 This control is exerted by the ExpR/Sin system via the *visNR* operon which codes for
16 the master regulator of flagellar, motility and chemotaxis genes. At low cell densities
17 ExpR is required for the activation of motility-related genes whereas at high population
18 densities ExpR in conjunction with AHLs inhibits transcription of the *visNR* operon,
19 resulting in the repression of genes belonging to the flagellar regulon (16).

20 It has been reported that swarming of *S. meliloti* depends on the presence of a
21 functional ExpR/Sin quorum sensing system (2,13). Two different laboratories have
22 described that only strains carrying a functional *expR* locus were able to swarm.
23 However, our recent data are in disagreement with these findings. We have reported that
24 the commonly used *S. meliloti* laboratory strain Rm1021 and the closely related strain
25 Rm2011, both known to have a disrupted copy of *expR*, are able to swarm on semisolid

1 minimal medium (32). To solve the discrepancies between these reports, in this work
2 we have re-examined the role played by the *expR* gene in swarming of *S. meliloti*. In
3 addition, we have extended our studies to investigate the role of exopolysaccharides
4 EPS I and EPS II on the surface motility of this bacterium.
5

6 MATERIALS AND METHODS

7 **Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids
8 used in this work and their relevant characteristics are listed in Table 1. *Escherichia coli*
9 strains were grown in Luria-Bertani (LB) medium (38) at 37ºC; *S. meliloti* strains were
10 grown at 30ºC either in complex tryptone yeast (TY) medium (4), in Bromfield medium
11 (BM) (0.04% tryptone, 0.01% yeast extract, and 0.01% $CaCl_2.2H_2O$) or in minimal
12 medium (MM) containing glutamate (6.5 mM), mannitol (55 mM), mineral salts
13 (K_2HPO_4, 1.3 mM; $KH_2PO_4 . 3H_2O$, 2.2 mM; $MgSO_4$ $7H_2O$, 0.6 mM; $CaCl_2$ $2H_2O$, 0.34
14 mM; $FeCl_3$ $6H_2O$, 0.022 mM; NaCl, 0.86 mM) and vitamins (biotin (0.2mg/L); calcium
15 pantothenate (0.1 mg/L)) (37). To detect overproduction of EPS I, calcofluor white
16 M2R (Fluorescent brightener 28, SIGMA) was added to TY or MM plates at a final
17 concentration of 0.02%. When required, antibiotics were added at the following final
18 concentrations (in µg ml^{-1}): streptomycin (50), spectinomycin (100), and kanamycin
19 (50), for *E. coli*; nalidixic acid (10), streptomycin (200), spectinomycin (100), rifampin
20 (100), kanamycin (200), neomycin (120), hygromycin (75 to 100) and oxytetracycline
21 (0.75) for *S. meliloti*. To improve reproducibility, all liquid cultures of *S. meliloti* were
22 routinely initiated from glycerol stocks. The ability of the different strains to grow in
23 liquid TY, BM and MM was monitored every two hours in a Bioscreen C apparatus (Oy
24 Growth Curves Ab Ltd, Finland).

Construction of *S. meliloti* strains. For the construction of *expR*$^+$ derivatives of Rm1021 (1021R) and Rm2011 (2011R), the functional *expR* gene of Rm8530 was PCR amplified using primers Rm*pyc* and Sm*ndvA2* (Table 2), cloned into pCR-XL-TOPO and sequenced. This construct was digested with EcoRI and the 1550 bp fragment containing the functional *expR* gene was isolated and subcloned into pK18*mobsacB* to yield plasmid pK18-expR. This plasmid was introduced into Rm1021 and Rm2011 via conjugation with S17-1, and allele replacement events were selected as described previously (39). In this case, clones in which allelic exchange occurred were easily identified as they showed a very noticeable mucoid phenotype. Strain QN1021 (*expR*) was obtained by replacing the disrupted *expR* locus of Rm1021 comprising the insertion sequence ISRm2011-1 and the IS-flanking loci *smc03896* and *smc03899* (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi) (34) with an unmarked deleted version. The *expR* deletion was generated *in vitro* by overlap extension PCR (20) using primers ExpR.1-ExpR.4 listed in Table 2. The resulting fusion product in which a deletion of 1943 bp was created, was cloned into pCR-XL-TOPO and sequenced. Using the HindIII and BamHI restriction sites included in the outside primers, the insert was subcloned into vector pK18*mobsacB* yielding plasmid pK18-ΔexpR. This construction was introduced into Rm1021 via conjugation with S17-1, and allele replacement events were selected as described previously (39). Likewise, *S. meliloti* mutant strains containing deleted versions of *exoX* and *exoY* were obtained by allelic replacement using the same methodology. The *exoX* and *exoY* mutant alleles harboring in frame deletions of 274 and 501 bp, respectively, were generated *in vitro* by overlap extension PCR using primers listed in Table 2. The resulting PCR products were cloned into pCR-XL-TOPO, sequenced, and by using the restriction sites included in the outside primers, subcloned into vector pK18*mobsacB* to yield plasmids pK18-

ΔexoX and pK18-ΔexoY. pK18-ΔexoX was introduced into Rm1021 and after selection of allele replacement, the EPS I-overproducer 1021X strain was obtained. pK18-ΔexoY was introduced into Rm1021, Rm11601 and 1021X to yield the corresponding mutant strains defective in EPS I (1021Y, 11601Y, and 1021XY, respectively). Phage ΦM12 transduction (10) was employed to transfer mutations amongst strains: i) The *flaA flaB* mutants 1021F (*expR flaA flaB*), 1021YF (*expR exoY flaA flaB*), and 1021XF (*expR exoX flaA flaB*) were obtained by transferring the Δ*flaA flaB*::Hy mutation from strain Rm11601 ($expR^+$ *flaA flaB*) to strains Rm1021 (*expR*), 1021Y (*expR exoY*), and 1021X (*expR exoX*), respectively; ii) The *flgE* mutants 2011RFg ($expR^+$ *flgE*) and 8530Fg ($expR^+$ *flgE*) were obtained by transferring the *flgE*::mini-Tn*5* mutation from strain 2011mTn5STM.1.03.E07 to 2011R ($expR^+$) and Rm8530 ($expR^+$), respectively; iii) The *rhbA* mutant 1021rhbA (*expR rhbA*) was obtained by transferring the *rhbA*::Tn*5lac* mutation from strain 2011rhbA62 to Rm1021 (*expR*); iv) Likewise, the *wgeB* mutants 8530W ($expR^+$ *wgeB*) and 11601W ($expR^+$ *flaA flaB wgeB*) were obtained by transferring the mini-Tn*5* disrupted locus *wgeB* from strain 2011mTn5STM.4.06.G01 to strains Rm8530 and Rm11601, respectively. All mutants constructed in this work were checked by PCR and Southern hybridization with specific probes.

Motility assays. Swimming was examined on plates prepared with BM containing 0.3% Bacto agar and inoculated with 3 µl droplets of rhizobial cultures grown in TY (OD_{600nm} = 1). Surface motility was analyzed using two different methodologies: i) The motility assay described by Bahlawane et al. (2) in which 3 µl of overnight TY rhizobial cultures were inoculated onto the surface of BM containing 0.6% Bacto agar, and ii) The motility test described in our previous work (32,42) in which 2 µl of washed 10-fold concentrated cultures grown in TY broth to the late exponential phase were inoculated onto semisolid MM plates. For swimming and surface motility tests

performed on BM, the migration zone was determined as the colony diameter in millimeters. In the case of surface motility tests performed on semisolid MM, in which fractal patterns with characteristic tendrils were formed, migration zones were calculated as the average length of the two sides of a rectangle exactly able to frame each colony.

CAS siderophore assay. The determination of siderophores in liquid cultures was performed using the Chrome azurol S (CAS) assay solution described by Schwyn and Neilands (40). Supernatants of *S. meliloti* cultures were mixed 1:1 with the CAS assay solution. After reaching equilibrium, the absorbance was measured at 630 nm.

RESULTS

ExpR promotes flagella-independent surface spreading of *S. meliloti*. Swimming motility tests performed with Rm1021 (*expR*), and Rm8530 (*expR*$^{+}$) revealed the ability of these strains to swim without significant differences amongst them (Fig. 1A), thereby confirming previously published results (2). The same strains were assayed for surface motility on 0.6% agar BM highlighting different phenotypes (Fig. 1B). Whereas macrocolonies formed by Rm1021 were dry and did not show signs of significant surface expansion after three days of incubation, those formed by Rm8530 were highly mucoid and clearly covered a larger surface area. However, neither the macroscopic appearance nor the slow translocation over the surface of BM shown by Rm8530 (Ca. 0.04 μm/s) was indicative of swarming motility. The two new Rm1021 derivative strains constructed in this study, QN1021 (*expR*) and 1021R (*expR*$^{+}$), showed the same behavior as Rm1021 (*expR*) and Rm8530 (*expR*$^{+}$), respectively. Bahlawane and coworkers described the surface expansion shown by their *S. meliloti expR*$^{+}$ strains as swarming based on the fact that Rm2011 *expR*$^{+}$-derivative strains defective in flagellum

production (*flgE* and *visN* mutants) were non-motile on semisolid BM (2). We tested the motility phenotype of three mutant derivative strains of Rm8530: Rm11601 (*expR*$^+$ *flaA flaB*) lacking functional flagellar filaments, 8530Vis (*expR*$^+$ *visN visR*) lacking the master regulator of flagellar, motility and chemotaxis genes, and 8530Fg (*expR*$^+$ *flgE*) affected in the gene putatively coding for the flagellar hook protein. These three mutant strains were devoid of flagella (16; data not shown) and consequently were incapable of swimming (Fig. 1A). On the contrary, on 0.6% agar BM, all three strains showed the same behavior as the flagellated parental strain Rm8530 (Fig. 1B). These results were in disagreement with the data presented by Bahlawane et al. in which non-flagellated *expR*$^+$ derivatives of the closely related strain Rm2011 were shown to be non-motile under the same conditions. To investigate if the differences were due to strain-specific effects, the motility of Rm2011 (*expR*) and that of its derivatives Sm2B3001 (*expR*$^+$), Sm2B5005 (*expR*$^+$ *flgE*) and Sm2B6005 (*expR*$^+$ *visN*), was tested on 0.6% agar BM (not shown in Fig. 1). The behavior of Rm2011 and the *visN* derivative mutant strain Sm2B6005 was reproducible in all our assays and in agreement with our results: colonies formed by the ExpR deficient strain Rm2011 were dry whereas those formed by the non-flagellated *expR*$^+$-derivative strain Sm2B6005 were highly mucoid and spread over the surface of semisolid BM significantly more than colonies formed by Rm2011 (9.9 ± 0.2 mm versus 5.4 ± 0.2 mm). On the contrary, an unstable mucoid phenotype was observed for strains 2B3001 (*expR*$^+$) and 2B5005 (*expR*$^+$ *flgE*), leading to unreliable results. Therefore, we decided to construct two new Rm2011 derivative strains: 2011R (*expR*$^+$) and 2011RFg (*expR*$^+$ *flgE*). As shown in Fig. 1B, the new strains behaved as Rm8530 (*expR*$^+$) and 8530Fg (*expR*$^+$ *flgE*). Altogether, these data demonstrate that *S. meliloti* strains harboring a functional *expR* gene are able to spread

on the surface of semisolid BM but the mechanism used is not dependent on flagella and therefore can not be described as swarming motility.

ExpR is not required for swarming motility of *S. meliloti*. When surface motility assays were performed on semisolid MM plates, surface translocation with characteristic tendril formation could be observed for the ExpR deficient strains Rm1021, QN1021 and Rm2011, already 14 to 20 h after inoculation (Fig 1C). To corroborate that the surface motility shown by *S. meliloti* ExpR deficient strains on semisolid MM was dependent on flagella, the motility phenotype of 1021F (*expR flaA flaB*), and 2011mTn5STM.1.03.E07 (*expR flgE*) was assayed and compared to their corresponding parental strains Rm1021 and Rm2011, respectively. As expected for non-flagellated bacteria, 1021F and 2011mTn5STM.1.03.E07 were non-motile in swimming assays performed in BM containing 0.3% agar (Fig. 1A). Likewise, as expected for ExpR deficient strains, they did not spread on 0.6% BM (Fig. 1B). On semisolid MM, surface translocation of these two strains was severely affected compared to their parental strains, although not completely abolished as is the case for 2011rhbA62 and 1021rhbA, mutant strains unable to produce the siderophore rhizobactin 1021, derived from Rm2011 and Rm1021, respectively (Fig. 1C and (32)). This result demonstrates that ExpR deficient *S. meliloti* strains are able to show flagella-driven surface translocation (i. e. swarming motility) on semisolid MM and therefore, we can conclude that ExpR is not required for swarming. The minor surface spreading shown by non-flagellated ExpR deficient strains reveals the existence of a second type of surface motility which is not dependent on flagellar activity. This ExpR and flagella-independent surface motility seems to be regulated by nutrient composition of media since it is manifested only on semisolid MM and not in semisolid BM. The fact that

1 gene mutations (*rhb*) and environmental conditions (high iron) which block the
2 synthesis of the siderophore rhizobactin 1021 render *S. meliloti* Rm1021/Rm2011
3 completely non-motile on semisolid MM (see 1021rhbA in Fig. 1C and (32)), suggests
4 that rhizobactin 1021 plays a role in both the flagella-driven as well as in flagella-
5 independent surface motilities shown by ExpR deficient *S. meliloti* strains. CAS assays
6 performed with supernatants of Rm1021/Rm2011 cultures grown in BM revealed the
7 lack of siderophore production (data not shown) which could explain the absence of
8 surface motility on semisolid BM by these ExpR deficient strains.

9 Surface motility assays on semisolid MM were also performed for *S. meliloti* strains
10 harboring a functional *expR* gene (Fig. 1C). In contrast to the behavior shown by
11 Rm1021/QN1021/Rm2011, colonies formed by 1021R/Rm8530/2011R (*expR*$^+$ strains)
12 were highly mucoid and showed smooth borders although some tendrils could also be
13 observed. Notably, the *expR*$^+$ strains spread extensively over the surface covering an
14 area which was almost twice as large as the area colonized by ExpR deficient strains,
15 suggesting that ExpR promotes surface translocation not only on semisolid BM but also
16 on semisolid MM. However, in contrast to *expR* mutant strains, the surface spreading
17 displayed by Rm8530 was not significantly reduced in the absence of flagella as
18 revealed by the phenotypes exhibited by Rm11601 (*expR*$^+$ *flaA flaB*), 8530Vis (*expR*$^+$
19 *visN visR*), and 8530Fg (*expR*$^+$ *flgE*) (Fig. 1C). Similar behavior was observed for
20 2011R and its non-flagellated derivative strain 2011RFg. These results indicate that, as
21 on semisolid BM, the surface translocation shown by *expR*$^+$ strains of *S. meliloti* on
22 semisolid MM is not dependent on flagellar activity and therefore can not be described
23 as swarming.
24

EPS II is not required for swarming and promotes sliding motility in *S. meliloti.* The observed correlation between mucoidy and flagella-independent surface motility shown by *expR*$^{+}$ strains, together with the role assigned to ExpR in EPS I and EPS II synthesis (21), prompted us to investigate the function of these exopolysaccharides in the surface spreading exhibited by *S. meliloti*.

It is known that ExpR-deficient strains of *S. meliloti*, such as Rm1021, do not produce EPS II at detectable levels unless they are grown under low phosphate conditions (29). Nevertheless, they are able to show swarming motility as we have demonstrated in this and previous work (32), indicating that EPS II is not required for this flagella-driven surface translocation. To investigate the role of EPS II on the motility of *S. meliloti expR*$^{+}$ strains we constructed *wgeB* (formerly *expE2*) mutants impaired in a glycosyl transferase involved in EPS II synthesis (3). Strains 8530W (*expR*$^{+}$ *wgeB*) and the non-flagellated 11601W (*expR*$^{+}$ *flaA flaB wgeB*) showed a nonmucoid phenotype in different media, as expected for *S. meliloti* strains unable to synthesize EPS II. Moreover, no relevant differences in swimming rings were detected between these strains and their corresponding parental strains (Rm8530 and Rm11601, respectively) (Fig. 2A), suggesting that EPS II plays no role in swimming motility. However, in contrast to their parental strains and all the *expR*$^{+}$ strains tested in this work, 8530W and 11601W did not spread over the surface of 0.6% BM, displaying the same phenotype as ExpR deficient strains (Fig. 2B). 8530W and 1161W did not show defects in growth in liquid BM (data not shown). Thus, these results clearly demonstrate that the flagella-independent surface translocation shown by ExpR strains of *S. meliloti* on semisolid BM is absolutely dependent on the production of EPS II. Therefore, this mode of translocation is most akin to sliding motility, whereby the production of EPS II promotes passive movement of cells across the agar surface.

The *wgeB* mutation also led to a significant reduction (23% in the case of flagellated Rm8530, and 37% for the non-flagellated Rm11601) in the surface motility shown by *expR*$^{+}$ strains of *S. meliloti* on semisolid MM (Fig. 2C). No differences in growth rates were detected in MM broth between the two *wgeB* mutants and their corresponding parental strains (data not shown). Therefore, also on MM, EPS II contributes to flagella-independent surface translocation or sliding which seems to be the predominant mode of translocation of *expR*$^{+}$ strains. Interestingly, when EPS II production is blocked, these strains exhibit swarming motility, manifested by the ca. 4.25 mm difference in surface spreading displayed by 8530W (*expR*$^{+}$ *wgeB*) (15.9 mm) and 11601W (*expR*$^{+}$ *flaA flaB wgeB*) (11.6 mm) which indeed is very similar to the difference in surface spreading (5 mm) shown by Rm1021 (*expR*) (12.2 mm) and 1021F (*expR*, *flaA flaB*) (7.2 mm). These results indicate that like in *expR* mutants, EPS II is not essential for the swarming motility of *expR*$^{+}$ strains. The sliding motility promoted by EPS II allows for a larger surface colonization than the swarming motility exhibited by the same strain when EPS II synthesis is blocked. This makes it difficult to determine if swarming and sliding coexist in *expR*$^{+}$ strains or if alternatively, EPS II production inhibits flagella-driven surface motility. In either case, our data revealed that, once EPS II production is impeded, ExpR does not significantly influence swarming motility in *S. meliloti*.

As is the case for the non-flagellated strain 1021F (*expR flaA flaB*), the ability of 11601W (*expR*$^{+}$ *flaA flaB wgeB*) to move over the surface of semisolid MM was not abolished. Indeed, the flagella-independent surface translocation shown by 11601W seemed to be enhanced compared to that shown by 1021F. This behavior could be the result of the better growth rate shown by 1161W in liquid MM compared to 1021F (data not shown). Regardless of the effect of growth on surface translocation, our results suggest that an ExpR-controlled factor might play a role in flagella-independent surface

translocation. It is tempting to speculate that this factor might be rhizobactin 1021 based on the role played by this siderophore in surface motility of ExpR deficient strains, and on the reported transcriptomic data which revealed higher expression of the *rhrA* gene (encoding the AraC-like regulator which positively regulates the production and transport of rhizobactin 1021) in Rm8530 (*expR*$^{+}$) than in Rm1021 (*expR*) (21). However this hypothesis has not been investigated here.

Overproduction of EPS I promotes both sliding and swarming motilities in ExpR deficient *S. meliloti* strains. To investigate the role of EPS I in the different types of motility shown by *S. meliloti*, several *exoY* mutants lacking a sugar transferase essential in EPS I synthesis (30), were generated. As shown in Fig. 2, under the three conditions tested, the phenotype exhibited by the *exoY* mutants 1021Y (*expR exoY*), 1021YF (*expR exoY flaA flaB*), Rm9020 (*expR*$^{+}$ *exoY*) and 11601Y (*expR*$^{+}$ *flaA flaB exoY*) was similar to that of their corresponding isogenic strains harboring a functional *exoY* locus Rm1021, 1021F, Rm8530 and Rm11601, respectively. These results demonstrate that the production of normal amounts of EPS I does not play a significant role in either swimming, swarming (observed on ExpR deficient strains on semisolid MM) or flagella-independent surface spreading (shown by *expR*$^{+}$ strains on both semisolid BM and MM, and by ExpR deficient strains on semisolid MM).

We decided to test if an increased production of EPS I could have an effect on motility, similar to the effect caused by the large amount of EPS II produced by *expR*$^{+}$ strains. The overproduction of EPS I was achieved by deleting most of the coding sequence of the *exoX* gene whose disruption has been shown to cause overproduction of low-molecular-weight EPS I in *S. meliloti* (36). The gene deletion eliminates essential amino acids required for the inhibitory effect of ExoX on exopolysaccharide synthesis,

an effect that it is thought to occur posttranslationally in a mechanism in which the stoichiometry with ExoY is important.

The *exoX* derivative mutant strains 1021X and 1021XF were more mucoid on MM plates than the corresponding parental strains Rm1021 and 1021F. Moreover, the higher fluorescence shown under long-wave UV light by 1021X and 1021XF grown on TY plates supplemented with the fluorescent dye Calcofluor, compared to their parental strains confirmed EPS I overproduction (data not shown). Motility tests performed with these strains revealed no significant differences in swimming (Fig. 2A). In addition, no surface translocation associated with the *exoX* mutation could be observed on semisolid BM (Fig. 2B). Interestingly, on semisolid MM, 1021X (*expR exoX*) that showed similar growth rate in liquid MM as the parental strain, exhibited the largest surface translocation of all the strains tested in this work, colonizing a surface area which was 2.4 fold wider than that of the parental strain Rm1021 (Fig. 2C). This movement was strongly diminished in the absence of flagella as revealed by the behavior of 1021XF (*expR exoX flaA flaB*), demonstrating that 1021X shows swarming motility. Furthermore, the flagella-promoted surface spreading exhibited by 1021X (*expR exoX*) (Ca. 16 mm) was approximately 3 fold larger than the flagella-driven surface translocation shown by Rm1021 (*expR*) (Ca. 5 mm) (Fig. 2C), indicating that overproduction of EPS I promotes swarming motility. On the other hand, EPS I-overproduction also promotes flagella-independent surface translocation on MM as revealed by the larger area colonized by 1021XF (*expR exoX flaA flaB*) compared to the surface area colonized by 1021F (*expR flaA flaB*) (Fig. 2C). Introducing an *exoY* mutation into the 1021X strain led to phenotypes (mucoidy, calcofluor brighteness, and surface motility) similar to those shown by Rm1021 (*expR*) and 1021Y (*expR exoY*)

1 (data not shown), demonstrating that the overproduction of EPS I was the only cause of

2 the observed effects in *exoX* mutants.

3

1 **DISCUSSION**

2 This work was aimed at solving the existing discrepancies concerning the role of the
3 LuxR type regulator ExpR in the swarming motility of *S. meliloti.* Two different groups
4 reported independently that swarming of *S. meliloti* depends on the presence of a
5 functional *expR* locus (2,13). However, we recently reported that strains Rm1021 and
6 Rm2011, both known to have a disrupted copy of *expR*, are able to swarm on semisolid
7 medium (32). We have re-examined the role played by ExpR by using different mutants
8 in different genetic backgrounds and assaying their motility phenotypes under the
9 experimental conditions described in the contradicting publications.

10 The new data showed that although ExpR deficient strains do not display surface
11 translocation on semisolid BM as it was reported by Bahlawane et al. (2) they exhibit
12 flagella-driven surface translocation on semisolid MM. Therefore, we can conclude that
13 ExpR is not essential for swarming motility. Moreover, it became clear that, as
14 previously reported for a *S. meliloti fadD* mutant (42), the swarming motility of ExpR-
15 deficient strains is greatly influenced by nutrient composition of media. Besides
16 flagella, the production of the siderophore rhizobactin 1021 which requires the presence
17 of low iron concentrations in the medium, is the only factor known up to now to play an
18 essential role in the swarming motility of ExpR deficient strains.

19 In addition to demonstrating the dispensability of ExpR for swarming motility in *S.*
20 *meliloti*, this study has unveiled the existence in *S. meliloti* of an additional mode of
21 surface translocation which does not require flagellar activity. This type of movement
22 was especially noticeable in strains harboring a functional *expR* locus in both semisolid
23 BM and MM. By using up to 4 different non-flagellated derivative mutants (including
24 those used in Bahlawanes's work), we clearly demonstrated that the surface spreading
25 shown by *expR*$^+$ strains on semisolid media was not significantly diminished by the

1 absence of flagella, and therefore cannot be considered swarming. However, when
2 synthesis of galactoglucan (EPS II) was blocked by generating *wgeB* mutations, surface
3 spreading of *expR*$^{+}$ strains was completely abolished on BM, and significantly reduced
4 on MM. Considering these data, the surface translocation shown by *expR*$^{+}$ strains of *S.*
5 *meliloti* is most akin to sliding motility (18,19), whereby the production of EPS II
6 promotes passive movement of cells across the agar surface.

7 To the best of our knowledge, this work represents the first report on sliding motility
8 in *Rhizobium*. Sliding or spreading by expansion has been described for a diverse group
9 of bacteria such as mycobacteria, *Bacillus subtilis*, *Vibrio cholerae, Serratia*
10 *marcescens, Pseudomonas aeruginosa* and *Legionella pneumophila* (1,5,9,27,31,43), in
11 which a strong correlation between sliding and the production of surfactants has been
12 established. For example, the production of rhamnolipids in *Pseudomonas*, the
13 lipopeptides surfactin and serrawettin in *Bacillus* and *Serratia*, respectively, or a
14 surfactant-like material in *Legionella* facilitate flagella-independent surface
15 translocation in these bacteria. Most of these surfactants also play a crucial role in
16 swarming motility (reviewed in (8,23,45)). We are not aware of the possible surfactant
17 properties of the galactoglucan produced by *S. meliloti* and we can only speculate about
18 its role in sliding motility. It might be possible that the high levels of EPS II excreted by
19 *expR*$^{+}$ strains serve either as a hydrated milieu that gives sufficient moisture to facilitate
20 the spreading of the colony or as a lubricant that reduces friction between cells and
21 surfaces. In any case and in contrast to surfactants such as rhamnolipids, surfactin or
22 serrawettin, EPS II is not essential for the swarming motility of *S. meliloti* as indicated
23 by the flagella-dependent translocation shown by EPS II-defective strains, regardless of
24 having or not a functional ExpR regulator.

1 In addition to swarming and EPS II-promoted sliding motility, *S. meliloti* strains can
2 also spread over surfaces, although to a lesser extent, using a flagella and EPS II-
3 independent type of motility. In ExpR deficient strains, this motility relies on the
4 production of the rhizobactin 1021 siderophore since abolishment of its synthesis
5 renders Rm1021/Rm2011 strains completely non-motile. Therefore, rhizobactin 1021
6 plays a crucial role in both swarming and flagella-independent surface translocation
7 shown by *expR* strains of *S. meliloti*. We recently observed that purified rhizobactin
8 1021 shows drop collapse activity (our unpublished results), a property probably
9 conferred by the presence of the long-chain fatty acid (E)-2-decenoic acid in its
10 chemical structure. Thus, it is very probable that, as reported for other surfactants which
11 play roles in swarming and sliding motilities, rhizobactin 1021 contributes to the surface
12 migration of *S. meliloti* by acting as a wetting agent. We have not demonstrated in this
13 work if rhizobactin 1021 also accounts for the flagella and EPS II-independent surface
14 translocation exhibited by *expR*$^+$ strains on semisolid MM, although this possibility is
15 very likely.
16 The results presented in this work also provide further insights into additional factors
17 contributing to surface translocation in *S. meliloti*. The phenotype exhibited by *exoX*
18 derivative mutants of Rm1021 demonstrate that the overproduction of EPS I, but not the
19 production of normal amounts of this EPS, facilitates both sliding and swarming
20 motilities. Extracellular polysaccharides have been involved in surface translocation in
21 other bacteria. Thus, the acidic capsular polysaccharide produced by *Proteus mirabilis*
22 known as colony migration factor (Cmf) which is an important component of the
23 extracellular matrix that surrounds swarmer cells, plays a key role in swarming motility
24 by reducing surface friction during translocation (17). A similar role could be attributed
25 to EPS I but only at high levels of production.

1 This work has unveiled the unexpected complexity of surface translocation in *S.*
2 *meliloti* raising questions that require further investigation. It is clear that EPS II-
3 dependent sliding is the most relevant type of surface translocation displayed by *expR*$^+$
4 strains of *S. meliloti*, allowing these bacteria to colonize surfaces more efficiently than
5 strains displaying only swarming motility. If swarming and sliding take place at the
6 same time in *expR*$^+$ strains or if EPS II inhibits swarming motility still remains unclear.
7 Furthermore, although we show here that ExpR is not required nor significantly
8 influences swarming motility in *S. meliloti*, we can not rule out the possibility of
9 population density regulation of swarming motility in this bacterium. Therefore, efforts
10 should be continued to identify and characterize other regulators and components which
11 play key roles in sliding and/or swarming. Another interesting question to be solved is
12 the role these types of surface motilities play in the different lifestyles of *Rhizobium*.
13 Whereas the role of swarming motility in the establishment of the *Rhizobium*-legume
14 symbiosis is still unclear, sliding motility may allow *S. meliloti* to colonize surfaces
15 under conditions where flagellar expression is down-regulated, for instance at high cell
16 population densities and during the invasion process. In line with this, a collective
17 sliding movement of bacteria toward the infection thread tip has been proposed to
18 contribute to colonization of the thread (11). The biological significance of the ability to
19 slide or swarm in the *Rhizobium*-legume symbiosis remains to be elucidated.
20

21 **ACKNOWLEDGEMENTS**

22 This work was supported by grants BIO2007-62988 and BIO2010-18005 from the
23 Ministerio de Ciencia e Innovación (Spain), CVI 03541 from the Junta de Andalucía
24 (Spain), and FEDER funds. JN and LBR were supported by grants from the Consejería
25 de Innovación, Ciencia y Empresa (Junta de Andalucía, Spain).

We are grateful to Juan E. González, Anke Becker and Birgit Scharf for providing several *S. meliloti* strains.

1 **REFERENCES**

2 1. **Agustí, G., O. Astola, E. Rodríguez-Güell, E. Julián, and M. Luquin**. 2008.
3 Surface spreading motility shown by a group of phylogenetically related, rapidly
4 growing pigmented mycobacteria suggests that motility is a common property of
5 mycobacterial species but is restricted to smooth colonies. J. Bacteriol. **190**:6894-
6 6902.

7 2. **Bahlawane, C., M. McIntosh, E. Krol, and A. Becker**. 2008. *Sinorhizobium*
8 *meliloti* regulator MucR couples exopolysaccharide synthesis and motility. Mol.
9 Plant Microbe Interact. **21**:1498-1509.

10 3. **Becker, A., S. Rüberg, H. Küster, A. A. Roxlau, M. Keller, T. Ivashina, H. P.**
11 **Cheng, G. C. Walker, and A. Pühler**. 1997. The 32-kilobase *exp* gene cluster of
12 *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic
13 organization and properties of the encoded gene products. J. Bacteriol. **179**:1375-
14 1384.

15 4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen.
16 Microbiol. **84**:188-198.

17 5. **Brown, I. I., and C. C. Häse**. 2001. Flagellum-independent surface migration of
18 *Vibrio cholerae* and *Escherichia coli*. J. Bacteriol. **183**:3784-3790.

19 6. **Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Dénarié**. 1979.
20 Identification and characterization of large plasmids in *Rhizobium meliloti* using
21 agarose-gel electrophoresis. J. Gen. Microbiol. **113**:229-242.

22 7. **Daniels, R., S. Reynaert, H. Hoekstra, C. Verreth, J. Janssens, K. Braeken,**
23 **M. Fauvart, S. Beullens, C. Heusdens, I. Lambrichts, D. E. De Vos, J.**
24 **Vanderleyden, J. Vermant, and J. Michiels**. 2006. Quorum signal molecules as
25 biosurfactants affecting swarming in *Rhizobium etli*. Proc. Natl. Acad. Sci. U. S. A
26 **103**:14965-14970.

27 8. **Daniels, R., J. Vanderleyden, and J. Michiels**. 2004. Quorum sensing and
28 swarming migration in bacteria. FEMS Microbiol. Rev. **28**:261-289.

29 9. **Fall, R., D. B. Kearns, and T. Nguyen**. 2006. A defined medium to investigate
30 sliding motility in a *Bacillus subtilis* flagella-less mutant. BMC. Microbiol. **6**:31.

31 10. **Finan, T. M., E. Hartweig, K. LeMieux, K. Bergman, G. C. Walker, and E. R.**
32 **Signer**. 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120-
33 124.

34 11. **Fournier, J., A. C. Timmers, B. J. Sieberer, A. Jauneau, M. Chabaud, and D.**
35 **G. Barker**. 2008. Mechanism of infection thread elongation in root hairs of
36 *Medicago truncatula* and dynamic interplay with associated rhizobial
37 colonization. Plant Physiol **148**:1985-1995.

38 12. **Fraser, G. M., and C. Hughes**. 1999. Swarming motility. Curr. Opin. Microbiol.
39 **2**:630-635.

13. **Gao, M., H. Chen, A. Eberhard, M. R. Gronquist, J. B. Robinson, B. G. Rolfe, and W. D. Bauer**. 2005. *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. J. Bacteriol. **187**:7931-7944.

14. **Glazebrook, J., and G. C. Walker**. 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56**:661-672.

15. **Glazebrook, J., and G. C. Walker**. 1991. Genetic techniques in *Rhizobium meliloti*. Methods Enzymol. **204**:398-418.

16. **Gurich, N., and J. E. González**. 2009. Role of quorum sensing in *Sinorhizobium meliloti*-Alfalfa symbiosis. J. Bacteriol. **191**:4372-4382.

17. **Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes**. 1995. A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. Mol. Microbiol. **17**:1167-1175.

18. **Harshey, R. M.** 2003. Bacterial motility on a surface: many ways to a common goal. Annu. Rev Microbiol. **57**:249-273.

19. **Henrichsen, J.** 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. **36**:478-503.

20. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease**. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51-59.

21. **Hoang, H. H., A. Becker, and J. E. González**. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. J. Bacteriol. **186**:5460-5472.

22. **Hoang, H. H., N. Gurich, and J. E. González**. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J. Bacteriol. **190**:861-871.

23. **Kearns, D. B.** 2010. A field guide to bacterial swarming motility. Nat. Rev. Microbiol. **8**:634-644.

24. **Kim, W., and M. G. Surette**. 2004. Metabolic differentiation in actively swarming *Salmonella*. Mol. Microbiol. **54**:702-714.

25. **Lynch, D., J. O'Brien, T. Welch, P. Clarke, P. O. Cuiv, J. H. Crosa, and M. O'Connell**. 2001. Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. J. Bacteriol. **183**:2576-2585.

26. **Marketon, M. M., M. R. Gronquist, A. Eberhard, and J. E. González**. 2002. Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel N-acyl homoserine lactones. J. Bacteriol. **184**:5686-5695.

1 27. **Matsuyama, T., A. Bhasin, and R. M. Harshey**. 1995. Mutational analysis of
2 flagellum-independent surface spreading of *Serratia marcescens* 274 on a low-
3 agar medium. J. Bacteriol. **177**:987-991.

4 28. **Meade, H. M., and E. R. Signer**. 1977. Genetic mapping of *Rhizobium meliloti*.
5 Proc. Natl. Acad. Sci. U. S. A **74**:2076-2078.

6 29. **Mendrygal, K. E., and J. E. González**. 2000. Environmental regulation of
7 exopolysaccharide production in *Sinorhizobium meliloti*. J. Bacteriol. **182**:599-
8 606.

9 30. **Müller, P., M. Keller, W. M. Weng, J. Quandt, W. Arnold, and A. Pühler**.
10 1993. Genetic analysis of the *Rhizobium meliloti exoYFQ* operon: ExoY is
11 homologous to sugar transferases and ExoQ represents a transmembrane protein.
12 Mol. Plant Microbe Interact. **6**:55-65.

13 31. **Murray, T. S. and B. I. Kazmierczak**. 2008. *Pseudomonas aeruginosa* exhibits
14 sliding motility in the absence of type IV pili and flagella. J. Bacteriol. **190**:2700-
15 2708.

16 32. **Nogales, J., Domínguez-Ferreras, C. V. Amaya-Gómez, van Dillewijn P., V.**
17 **Cuéllar, J. Sanjuán, J. Olivares, and M. J. Soto**. 2010. Transcriptome profiling
18 of a *Sinorhizobium meliloti fadD* mutant reveals the role of rhizobactin 1021
19 biosynthesis and regulation genes in the control of swarming. BMC. Genomics
20 **11**:157.

21 33. **Overhage, J., M. Bains, M. D. Brazas, and R. E. Hancock**. 2008. Swarming of
22 *Pseudomonas aeruginosa* is a complex adaptation leading to increased production
23 of virulence factors and antibiotic resistance. J. Bacteriol. **190**:2671-2679.

24 34. **Pellock, B. J., M. Teplitski, R. P. Boinay, W. D. Bauer, and G. C. Walker**.
25 2002. A LuxR homolog controls production of symbiotically active extracellular
26 polysaccharide II by *Sinorhizobium meliloti*. J. Bacteriol. **184**:5067-5076.

27 35. **Pobigaylo, N., D. Wetter, S. Szymczak, U. Schiller, S. Kurtz, F. Meyer, T. W.**
28 **Nattkemper, and A. Becker**. 2006. Construction of a large signature-tagged
29 mini-Tn*5* transposon library and its application to mutagenesis of *Sinorhizobium*
30 *meliloti*. Appl. Environ. Microbiol. **72**:4329-4337.

31 36. **Reed, J. W., M. Capage, and G. C. Walker**. 1991. *Rhizobium meliloti exoG* and
32 *exoJ* mutations affect the ExoX-ExoY system for modulation of
33 exopolysaccharide production. J. Bacteriol. **173**:3776-3788.

34 37. **Robertsen, B. K., P. Aiman, A. G. Darwill, M. Mcneil, and P. Albersheim**.
35 1981. The structure of acidic extracellular polysaccharides secreted by *Rhizobium*
36 *leguminosarum* and *Rhizobium trifolii*. Plant Physiol **67**:389-400.

37 38. **Sambrook, J., and D. R. Russell.** 2001. Molecular Cloning: a Laboratory
38 Manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

39 39. **Schäfer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Pühler**.
40 1994. Small mobilizable multi-purpose cloning vectors derived from the

Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69-73.

40. **Schwyn, B., and J. B. Neilands**. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47-56.

41. **Simon, R., U. Priefer, and A. Pühler**. 1983. A broad host range mobilization system for *in vivo* genetic-engineering: transposon mutagenesis in Gram-negative bacteria. Biotech. **1**:784-791.

42. **Soto, M. J., M. Fernández-Pascual, J. Sanjuán, and J. Olivares**. 2002. A *fadD* mutant of *Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. Mol. Microbiol. **43**:371-382.

43. **Stewart, C. R., O. Rossier, and N. P. Cianciotto**. 2009. Surface translocation by *Legionella pneumophila*: a form of sliding motility that is dependent upon type II protein secretion. J. Bacteriol. **191**:1537-1546.

44. **Tambalo, D. D., C. K. Yost, and M. F. Hynes**. 2010. Characterization of swarming motility in *Rhizobium leguminosarum* bv. viciae. FEMS Microbiol. Lett. **307**:165-174.

45. **Verstraeten, N., K. Braeken, B. Debkumari, M. Fauvart, J. Fransaer, J. Vermant, and J. Michiels**. 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol. **16**:496-506.

46. **Wang, Q., J. G. Frye, M. McClelland, and R. M. Harshey**. 2004. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. Mol. Microbiol. **52**:169-187.

1 **FIGURE LEGENDS**

2 **Figure 1.**- Role of ExpR in motility of *S. meliloti*. (A) Swimming test in Bromfield
3 (0.3% agar). (B) Surface motility on semisolid Bromfield medium (0.6% agar). (C)
4 Surface motility on semisolid MM (0.6% agar). Relevant genotype is indicated under
5 the name of each strain. Pictures were taken 2 days (A), 3 days (B) or 20 hours (C) after
6 inoculation. Under each image, the mean and standard deviation of migration zones
7 (given in millimeters and measured as described in the text) obtained from at least nine
8 measurements is indicated.

9

10 **Figure 2.**- Role of exopolysaccharides EPS I and EPS II in motility of *S. meliloti*.
11 (A) Swimming test in Bromfield (0.3% agar). (B) Surface motility on semisolid
12 Bromfield medium (0.6% agar). (C) Surface motility on semisolid MM (0.6% agar).
13 Relevant genotype is indicated under the name of each strain. Pictures were taken 2
14 days (A), 3 days (B) or 20 hours (C) after inoculation. Under each image, the mean and
15 standard deviation of migration zones (given in millimeters and measured as described
16 in the text) obtained from at least nine measurements is indicated.

17

1 **TABLE 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
S. meliloti strains		
Rm2011	Wild type; Nx^r Sm^r	(6)
Rm1021	SU47 *expR102*::IS*Rm*2011-1; Sm^r	(28)
Rm8530	Rm1021 *expR*$^+$; Sm^r	(14)
2011m..E07	2011mTn5STM.1.03.E07, Rm2011 *flgE*::mini-Tn*5*; Nx^r Sm^r Nm^r	(35)
2011mTn5STM.4.06.G01	Rm2011 *wgeB*::mini-Tn*5*; Nx^r Sm^r Nm^r	(35)
Sm2B3001	Rm2011 with a restored *expR* gene; Nx^r Sm^r	(2)
Sm2B5005	Sm2B3001 *flgE*::mini-Tn*5*; Nx^r Sm^r Nm^r	(2)
Sm2B6005	Sm2B3001 *visN*::Spec^r; Nx^r Sm^r Spec^r	(2)
2011R	Rm2011 with a restored *expR* gene; Nx^r Sm^r	This study
2011RFg	2011R *flgE*::mini-Tn*5*; Nx^r Sm^r Nm^r	This study
2011rhbA62	Rm2011 *rhbA*::Tn*5lac*; Sm^r Rif^r Nm^r	(25)
QN1021	Rm1021 with a full deleted *expR* locus; Sm^r	This study
1021F	Rm1021 *flaA flaB*; Sm^r Hy^r	This study
1021R	Rm1021 with a restored *expR* gene; Sm^r	This study
1021rhbA	Rm1021 *rhbA*::Tn*5lac*; Sm^r Nm^r	This study
1021Y	Rm1021 Δ*exoY*; Sm^r	This study

1021YF	1021Y *flaA flaB*; Sm^r Hy^r	This study
1021X	Rm1021 Δ*exoX*; Sm^r	This study
1021XF	1021X *flaA flaB*; Sm^r Hy^r	This study
1021XY	1021X Δ*exoY*; Sm^r	This study
Rm11601	Rm8530 *flaA flaB*; Sm^r Hy^r	(16)
8530Vis	Rm8530 with full deletion of *visN visR*; Sm^r	B. Scharf
8530Fg	Rm8530 *flgE*::mini-Tn*5*; Sm^r Nm^r	This study
Rm9020	Rm8530 *exoY*::Tn*5-132*; Sm^r Otc^r	(15)
11601Y	Rm11601 Δ*exoY*; Sm^r Hy^r	This study
8530W	Rm8530 *wgeB*::mini-Tn*5*; Sm^r Nm^r	This study
11601W	Rm11601 *wgeB*::mini-Tn*5*; Sm^r Nm^r	This study
Escherichia coli strains		
DH5α	*supE44*, Δ*lacU169*, Φ80, *lacZ*ΔM1, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*, *5hsdR171*	Bethesda Research Lab®
S17.1	*thi*, *pro*, *recA*, *hsdR*, *hsdM*, Rp4Tc::Mu, Km::Tn*7*; Tp^r, Sm^r, $Spec^r$	(41)
Plasmids		
pCR-XL-TOPO	Cloning vector; Km^r	Invitrogen
pK18*mobsacB*	Suicide plasmid; Km^r	(39)
pK18-ΔexpR	pK18*mobsacB* carrying the deleted version of the *expR* locus; Km^r	This study
pK18-expR	pK18*mobsacB* carrying the *expR* gene from Rm8530; Km^r	This study

pK18-ΔexoY	pK18*mobsacB* carrying the deleted version of the *exoY* locus; Km^r	This study
pK18-ΔexoX	pK18*mobsacB* carrying the deleted version of the *exoX* locus; Km^r	This study

1

2 [a] Nx^r Sm^r Nm^r Spec^r Rif^r Hy^r Otc^r Tp^r Km^r: nalidixic acid, streptomycin, neomycin,

3 spectinomycin, rifampin, hygromycin, oxytetracycline, trimethoprim, and kanamycin

4 resistance, respectively.

5

6

1

2 **TABLE 2.** Primers used in this study

Primer	Sequence (5´ to 3´)[a]	Used for
Rm*pyc*	AGAGTGGCGTGAACATTCGG	*expR* restoration
Sm*ndvA2*	TCCTTCTGTGACGAGATCG	*expR* restoration
ExpR.1	AAAAAGCTTGCTTTTCGAGATAGACCTCG (HindIII)	*expR* deletion
ExpR.2	CGTACAGTTTCTGGCTGGTACATGAACG	*expR* deletion
ExpR.3	CGTTCATGTACCAGCCAGAAACTGTACGAGC	*expR* deletion
ExpR.4	AAAGGATCCCGTGAACTTCTTCAGTTCGC (BamHI)	*expR* deletion
del*exoY*.1	AAAGGATCCACCTCATAAGAGTTGTTGCC (BamHI)	*exoY* deletion
del*exoY*.2	GGACATATTGCGTGTTTGCCATACCTCC	*exoY* deletion
del*exoY*.3	GGAGGTATGGCAAACACGCAATATGTCC	*exoY* deletion
del*exoY*.4	AAAGGATCC AATACCGTCAAATTGGGAGC (BamHI)	*exoY* deletion
exoX1	AATAAGCTTGGACTTCATAGAGGTGACTC (HindIII)	*exoX* deletion
exoX2	GCTCAGGAATTGAGGGTGCGAACATGGC	*exoX* deletion
exoX3	GCCATGTTCGCACCCTCAATTCCTGAGCGGC	*exoX* deletion
exoX4	AATGGATCCGAGCGTAGAGATCGTAATC (BamHI)	*exoX* deletion

3

4 [a] Restriction sites used for cloning (underlined) are given in parenthesis

5

Figure 1

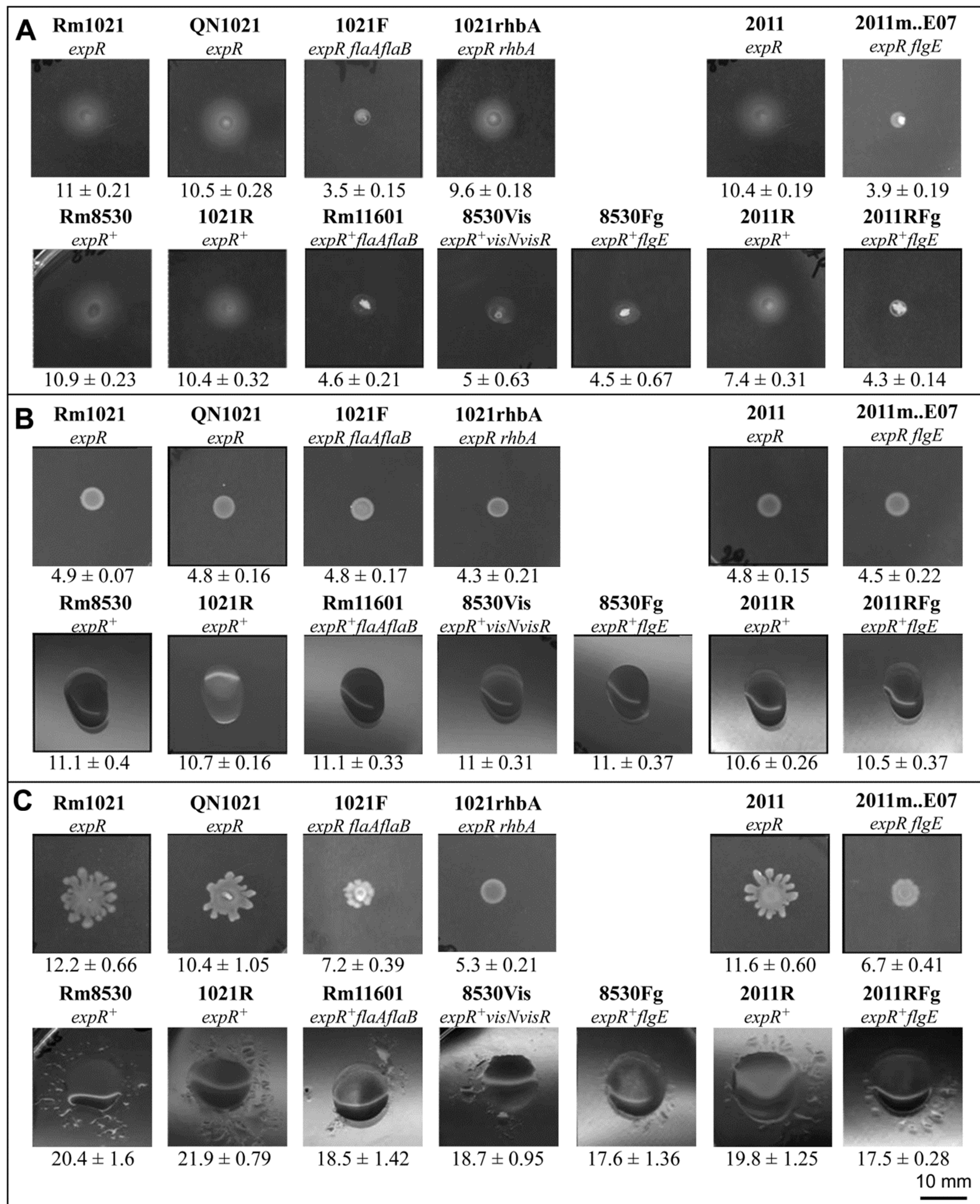

Figure 2