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yozG is needed for swarming in the undomesticated *Bacillus subtilis* strain *NCIB 3610*

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Abstract: Bacillus subtilis *is capable of swarming motility on semisolid surfaces. Here we characterize the swarming phenotype of a mutant in the gene of unknown function,* yozG *in the undomesticated strain 3610. The* yozG *mutant was unable to swarm. Swarming could be restored to the mutant by overexpression of the swarming regulator gene* swrA *or by overexpression of the flagella and chemotaxis operon. In addition, we were able to isolate two genetic suppressors of the* yozG *mutant that could also restore swarming.* yozG *is necessary for swarming in* B. subtilis *and likely acts upstream of the swarming regulator, SwrA.*

Introduction

Swarming motility is the coordinated, flagella-powered movement of groups of bacteria across a semisolid surface (1–3). Swarming is genetically distinct from swimming motility, requiring the production of extra flagella (hyperflagellation) and secretion of a surfactant (1, 4–6). In the undomesticated strain of *Bacillus subtilis* (NCIB 3610), broth-grown cells inoculated onto the center of a swarming plate will remain at the point of inoculation for a period of time termed the lag period before rapidly swarming across the surface of the agar (7–9). The lag period may represent the time required for cells to sense surface contact and respond, since cells taken from the edge of a swarm do not lag (7). The transition from swimming motility to swarming motility is governed by the master regulator SwrA (8–10). Other proteins, such as LonA and DegU, control SwrA abundance and activity (11–15). When *B. subtilis* are plated on semisolid media, SwrA levels increase, resulting in overexpression of the flagella and chemotaxis operon, *fla/che*. Overexpression of *fla/che* results in production of the extra flagella needed for swarming (7, 8, 10).

Here we characterize the function of the gene *yozG*. *yozG* encodes a predicted 84 amino acid-long protein, YozG, containing a helix-turn-helix motif (16). YozG is a cro/C1 type transcriptional regulator belonging to the XRE family which are involved in virulence, toxin/antitoxin systems, and stress response (16–20).

Mutation of *yozG* resulted in a loss of swarming motility, but not swimming motility, in the undomesticated strain *Bacillus subtilis* NCIB 3610. Here we show that mutation of *yozG* can be bypassed by overexpression of the master regulator *swrA*, suggesting that *yozG* acts upstream of *swrA* in the regulatory cascade.

Methods

Media and growth conditions

Unless otherwise specified, *B. subtilis* cultures were grown from frozen stocks on Luria Burtani (LB) plates (10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 5 g NaCl per liter) solidified with 1.5% Bacto agar at 37°C overnight. Liquid cultures were produced by subculturing a single colony from the LB plates into LB broth, and were agitated in a New Brunswick Scientific Excella E24 shaking incubator at 150-200 RPM at either 27 or 37°C as specified in each experiment.

For floating pellicle biofilm assays, *B. subtilis* strain NCIB 3610 was grown in minimal salts glutamate glycerol (MSgg) broth (95.37 mL sterile MQ water, 7.5 mL 0.1 M Phosphate Buffer, 30 mL 0.5 M MOPS pH 7, 3 mL 0.1 M $MgCl_2$, 1.05 mL 0.1 M $CaCl_2$, 0.75 mL 0.01 M $MnCl_2$, 0.9 mL 8.35 mM $FeCl_3$, 0.15 mL 1.0 mM $ZnCl_2$, 0.03 mL 0.01 M thiamine HCl, 1.5 mL 50% glycerol, 7.5 mL 10% glutamic acid, 0.75 mL 10 mg/mL tryptophan, 0.75 mL 10 mg/mL phenylalanine, and 0.75 mL 10 mg/mL threonine per 150 mL media).

Strain Construction

Strains used in this study are listed in Table 1. Primers are listed in Table 2.

Genetic markers were moved between strains using SPP1-mediated generalized phage transduction (21). To perform transduc-

Table 1. Strains used in this study.

Strain	Genotype	Notes	Source	Alternative name
DK1042*	3610 comIQ12L		D.B. Kearns (IUB), (32)	
BKE18740	168 yozG::erm		BGSC‡, (33)	
JS42	DK1042 *yozG::erm*	DK1042 x BKE18740	D.B. Kearns (IUB)	DK3375
JS43	DK1042 *amyE::*$P_{hyspank}$*-yozG spec*		D.B. Kearns (IUB)	DK3376
JS44	JS42 *swr+ suppressor 1*		this work	
JS46	JS42 *swr+ suppressor 2*		this work	
JS51	DK1042 *yozG::erm amyE::*$P_{hyspank}$*-yozG spec*	JS43 x JS42	this work	
JS61	3610 $P_{fla/che}\Omega P_{hyspank}$*-fla/che kan*		D.B. Kearns (IUB), (34)	DK29
JS57	3610 *amyE::*$P_{hyspank}$*-swrA spec*		D.B. Kearns (IUB), (26)	DS860
JS67	DK1042 *yozG::erm amyE::*$P_{hyspank}$*-swrA spec*	JS42 x JS57	this work	
JS69	DK1042 *yozG::erm* $P_{fla/che}\Omega P_{hyspank}$*-fla/che kan*	JS42 x JS61	this work	
JS75	168 *yoaS::kan*		BGSC‡, (33)	BKK18730
JS77	168 *yoaT::kan*		BGSC‡, (33)	BKK18750
JS79	3610 *yoaS::kan*	3610 x JS75	this work	
JS81	3610 *yoaT::kan*	3610 x JS77	this work	

*DK1042 is an undomesticated strain of *B. subtilis* derived from NCIB 3610. DK1042 lacks a plasmid-encoded competence inhibitor and is therefore more easily genetically manipulated than NCIB 3610. DK1042 and 3610 swarm identically and are both considered wild type for swarming (32, 35, 36). Notes indicate the recipient and donor (recipient x donor) used for SPP1 mediated phage transduction to construct the strain.
‡Bacillus Genetic Stock Center (bgsc.org).

tions, donor strains were grown in TY broth (LB broth supplemented with 10mM $MgSO_4$ and 0.1mM $MnSO_4$) until turbid. SPP1 phage stock was serially diluted in TY broth, and 0.1 mL of serial dilutions were mixed with 0.2 mL of turbid culture and incubated statically at 37°C for 15 minutes. Three mL of molten TY soft agar (TY broth with 0.5% w/v Bacto agar) was added to each tube and immediately mixed by rolling the tube between two hands and poured onto TY agar plates. Plates were dried open faced in a laminar flow hood for 20 minutes and then incubated overnight at 30°C. The next day, plates were examined and the plate with nearly confluent plaques was selected for harvesting. Phage were harvested by pipetting 5 mL of TY broth on top of the plate and scraping off the soft agar with a sterile cell scraper into a conical centrifuge tube. The tube was vortexed to liberate the phage from the soft agar and then centrifuged at 5000g for 10 minutes to pellet the agar. The supernatant was then filtered through a 0.45 μm syringe filter to isolate phage. Recipient cells were grown in 3 mL TY broth at 37°C until turbid, and 1 mL of turbid culture was mixed with 10-100 μL of phage stock in a 15 mL conical tube. Sterile TY media (9 mL) was added and the tubes were incubated at 37°C for 30 minutes. Tubes were centrifuged at 5000g for 10 minutes. The supernatant was poured off and the cell pellet was resuspended in the residual volume before plating 100 μL on LB media supplemented

Table 2. Primers used in this study

Primer name	Primer sequence
OAM010	ATGGACACAACAACAGCAAAACAGGC
OAM009	GAAAACAATAAACCCTTGCATAGGGGGATCGGGCAAGGCTAGACGGGACTTACC
OAM14	GATCCCCCTATGCAAGGGTTTATT
OAM13	AGTAGTTCCTCCTTATGTAAGC
OEA191	GGATAACAATTAAGCTTACATAAGGAGGAACTACTATGGCGATTATTATCAACATTGAT
OEA192	TTCCACCGAATTAGCTTGCATGCGGCTAGCGCCAATCACTTTATTTTTCTGTTG
OAM11	TAATGGATTTCCTTACGCGAAATA
OAM12	GCTAGCCGCATGCAAGCTAATT
OAM002	CCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTATGTACTATTTCGATCAGACCAG
OAM001	AGAAGCGTTAGCGGCAGCAAGTGAT
OJH001	CATATGTAAGATTTAAATGCAACCG
OJH002	CTACAAGGTGTGGCATAATGTGT

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with 10 mM sodium citrate and appropriate antibiotic to select for the antibiotic marker being moved and incubated at 37°C overnight. When appropriate, antibiotics were added to the following final concentrations: 5 µg/mL kanamycin (kan), 100 µg/mL spectinomycin (spec), and 1µg/mL erythromycin (erm).

yozG::erm

Strain JS42 was produced by SPP1 mediated phage transduction using strain BKE18740, which contains the yozG reading frame disrupted by an erythromycin resistance cassette, as a donor and DK1042 as a recipient. Transductants were plated and selected on LB agar containing 1 µg/mL erythromycin.

amyE::$P_{hyspank}$-yozG spec

The yozG overexpression construct was made using Gibson assembly. *amyE* UP and DOWN fragments were PCR amplified using primer pairs OAM010/OAM009 and OAM001/OAM002, respectively using *B. subtilis* genomic DNA as a template. The Spectinomycin resistance cassette and $P_{hyspank}$ promoter and ribosome binding site were amplified from pDR111 using primer pair OAM10 and OAM13. *yozG* was amplified from the genome using primer pair OEA191 and OEA192. *lacI* was amplified from pDR111 using primer pair OAM11 and OAM12. The final assembly was *amyE*UP-*spec*-$P_{hyspank}$-optRBS-*yozG*-*lacI*-*amyE*DOWN. Assembled fragments were transformed into *B. subtilis* strain 168 and plated on LB supplemented with 100 µg/mL spectinomycin. Single colonies were streaked for isolation on LB agar supplemented with 100 µg/mL spectinomycin and patched on starch containing plates to check for integration at the amyE locus. Final insertion was confirmed by PCR with primers OJH001 and OJH002.

Motility Assays

For quantitative swarm expansion assays, *B. subtilis* was grown in LB broth until OD_{600} was between ~0.5 and 1.0. One mL of culture was harvested via centrifugation, the supernatant was removed, and the cell pellet was resuspended to a calculated OD_{600} of 10 in PBS-India Ink buffer (5 µL India Ink, 1 mL 1x Phosphate buffered saline buffer). A 10 µL drop of the cell suspension was inoculated onto the center of agar plates containing 25 mL of LB broth solidified with 0.7% Bacto agar which had been prepared the previous day and had been let to dry for 20 minutes open-faced in a laminar flow hood. Plates were dried an additional 10 minutes open-faced in a laminar flow hood post inoculation. Plates were incubated at 37°C and the swarm radius was measured every 30 minutes along a transect from the inoculation point.

For qualitative swarm expansion assays, LB swarm plates were prepared as for quantitative assays, dried 20 minutes, and a single colony of *B. subtilis* was inoculated as a single point onto the surface of the agar in the center of the plate using a sterile wooden stick. Plates were dried an additional 10 minutes and incubated at 37°C overnight. Plates were photographed against a black background.

For qualitative swim assays, LB plates were prepared and solidified with 0.3% agar. Drying and inoculation of plates was performed as for qualitative swarm assays.

Growth Curves

Two milliliter overnight LB cultures were grown from single colonies of bacteria. The OD_{600} of the overnight cultures was determined and used to inoculate 25 mL of LB broth in 250 mL baffle flasks to a calculated OD_{600} of 0.01. Flasks were incubated at 37°C with shaking at 150 rpm. Samples of 800 µL from each flask were removed every 30 minutes and the OD_{600} was measured using a Spectronic 200 Spectrophotometer.

Statistical analysis

For swarm expansion assays and growth curves, analysis was focused on the linear portion of the swarm assays (eg. timepoints between ~2 and 4 hours). Standard analysis of covariance were used to simultaneously test the effect of strain, time, and the interaction of strain and time on swarm expansion or culture OD_{600} for each strain. Swarm radius and OD_{600} was expected to change with time, thus significant regression effects were ignored. Evaluation of the interaction effect for each model allowed us to determine if different strains within an experiment expanded or increased in optical density at different rates over the time course of measurement; e.g. a significant interaction effect indicated that strains generally exhibited different rates without pinpointing particular pairwise differences in rate. For strains that exhibited the same swarming rate, ANCOVA analysis revealed if the Y-intercepts for the swarms were significantly different, indicating a difference in the timing of swarm initiation.

Results

yozG is needed for wild-type swarming behavior

A colleague (Dr. Daniel Kearns) sent us a *yozG* mutant (JS42, Table 1) with a suspected motility defect in the undomesticated, swarming proficient NCIB 3610 strain of *Bacillus subtilis*. The mutant was an allelic replacement in which the reading frame of the *yozG* gene was disrupted by an antibiotic resistance cassette, originally obtained from the Bacillus Genetic Stock Center and transduced into the 3610 background by Dr. Kearns. To confirm the defect, my lab tested the swimming and swarming phenotype of the strain. While the *yozG* mutant was swimming proficient when inoculated onto 0.3% agar LB swim plates (data not shown), the mutant failed to swarm over the surface of 0.7% agar LB swarm plates and remained constricted to a small area around the point of inoculation (Figure 1A, 1B).

yozG is the second gene in a three-gene operon *yoaS*-*yozG*-*yoaT* (Figure 2). A putative small RNA, *S703,* is also encoded between the 3′ end of *yozG* and the 5′ end of *yoaT* (Figure 2). To determine if mutation of *yoaS* or *yoaT* also affected swarming, we procured allelic replacement mutants of *yoaS* and *yoaT* from the Bacillus Genetic Stock Center and

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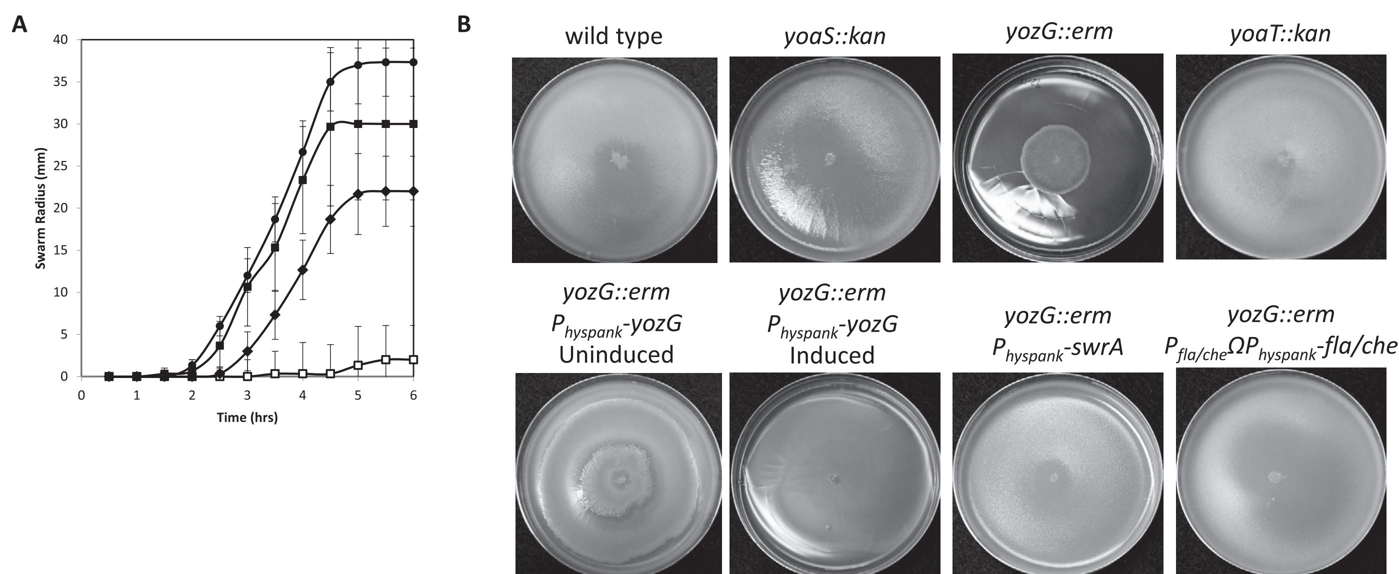
Figure 1. *yozG* mutants fail to swarm. A. Wild type (DK1042, filled circles), *yozG* mutant (JS42, *yozG::erm*; empty squares), complementation with IPTG inducer added (JS51, *yozG::erm amyE::$P_{hyspank}$-yozG spec;* filled squares), and complementation without IPTG inducer added (JS51, *yozG::erm amyE::$P_{hyspank}$-yozG spec;* filled diamonds), strains were inoculated onto the center of 0.7% agar LB swarm plates with or without 0.1 mM IPTG and incubated at 37°C. Swarm radius was measured along a transect of the plate every 30 minutes. Lines represent the average of three replicates. Error bars represent twice the standard error of the mean. B. Wild type (DK1042), *yoaS, yozG*, and *yoaT* mutants (JS79, JS42, JS80, respectively), complementation (JS51) without and with IPTG inducer added, and *yozG* mutant plus *swrA* overexpression or *fla/che* overexpression (JS67 and JS69, respectively) strains were inoculated on the center of swarm plates and incubated at 37°C overnight. Plates were photographed on a black background so that colonized agar appears light, and uncolonized agar appears dark. Photos are representative examples of three replicates.



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moved the alleles into our swarming-proficient wild-type background. Allelic replacement of *yoaS* or *yoaT* did not result in any change in swarming behavior, and we conclude that only the *yozG* gene in the operon is needed for swarming motility (Figure 1B).

To confirm that the nonswarming phenotype of the *yozG* mutant was due to disruption of the *yozG* reading frame, we complemented the mutant. The nonswarming phenotype of the *yozG* mutant could be rescued via complementation with a copy of *yozG* under the control of an inducible promoter at an ectopic locus (Figure 1A, filled squares). The complemented strain swarmed at a similar rate to wild type—the slope of the linear portion of the wild type swarm was 15, and of the complemented strain was 13. Interestingly, swarming was partially restored to the mutant even when inducer was not added to the growth medium, but the calculated slope of the linear portion was only 9.3 (Figure 1A, filled diamonds). The swarming rates of the three strains were still significantly different in our analysis ($p = 1.2e^{-2}$).

We cannot fully explain the partial phenotype rescue in the absence of inducer. However, we infer that very low levels of *yozG* are necessary in the cell, and evidence from SubtiWIki indeed shows low levels of *yozG* expression in most conditions (22). In support of this hypothesis, the swarming exhibited a bullseye pattern in our qualitative swarm assay (Figure 1B). Such patterning is evidence of consolidation, in which swarming cells dedifferentiate into nonswarming cells (8, 23). The leaky expression from the uninduced complementation of *yozG* could be supplying enough *yozG* to support swarming, but is unable to support a confluent swarm. We suspect a

Figure 2. Diagram of the *yoaS-yozG-yoaT* operon. *yoaS, yozG,* and *yoaT* comprise a three gene operon with a putative small RNA, S702. Gene lengths are relative but not to exact scale.

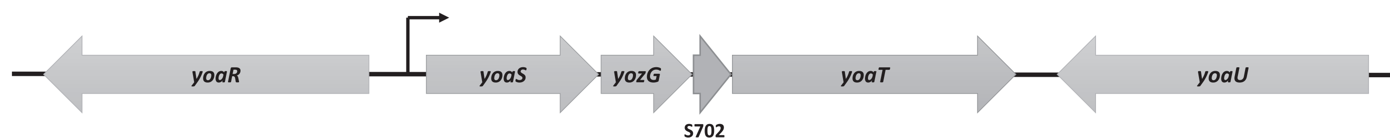
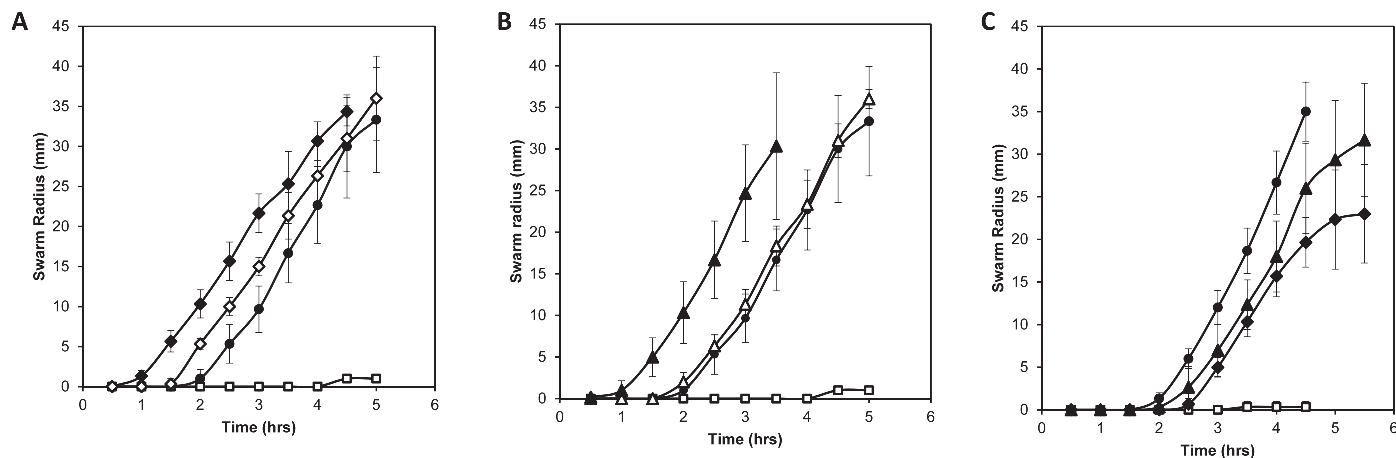


Figure 3. Overexpression of *fla/che* or *swrA* restores swarming to the *yozG* mutant. Swarm expansion assays in which strains were inoculated onto the center of 0.7% agar LB swarm plates (containing 0.1 mM IPTG for parts A and B) and incubated at 37°C. Swarm radius was measured along a transect of the plate every 30 minutes. Lines represent the average of three replicates. Error bars represent twice the standard error of the mean. A. Wild type (DK1042, filled circles), *yozG* mutant (JS42, *yozG::erm*; empty squares), *fla/che* overexpression (JS61, $P_{fla/che}\Omega P_{hyspank}$-*fla/che;* filled diamonds), and *yozG* mutant plus *fla/che* overexpression (JS69, *yozG::erm* $P_{fla/che}\Omega P_{hyspank}$-*fla/che;* empty diamonds). B. Wild type (DK1042, filled circles), *yozG* mutant (JS42, *yozG::erm*; empty squares), *swrA* overexpression (JS57, $P_{hyspank}$-*swrA;* filled triangles), and *yozG* mutant plus *swrA* overexpression (JS67, *yozG::erm* $P_{hyspank}$-*swrA;* empty triangles). C. Wild type (DK1042, filled circles), *yozG* mutant (JS42, *yozG::erm*; empty squares), and two spontaneous suppressors (JS44 and JS46, filled diamonds and filled triangles, respectively).



growth phenotype, discussed later, is effecting the swarming of the yozG complement both with and without induction.

Mutation of yozG can be bypassed by overexpression the master regulator, swrA

Swarming in undomesticated *B. subtilis* is controlled by the master regulator SwrA, encoded by *swrA* (8–10). SwrA increases expression of the flagella and chemotaxis operon, *fla/che,* resulting in hyperflagellation, and is essential for swarming motility (7–10, 14, 15). Overexpression of either *swrA* or the *fla/che* operon results in swarming without a lag period (8–11, 15, 24). We wanted to determine if the swarming defect of the *yozG* mutant could be bypassed by either overexpression of the flagella and chemotaxis operon, *fla/che,* or by overexpression of *swrA*. We placed an inducible promoter upstream of the *fla/che* operon and introduced our *yozG* mutation into that strain. Whereas induction of the *fla/che* promoter in a wildtype background resulted in the expected lagless swarming phenotype, induction of the *fla/che* promoter in our *yozG* mutant background restored wild type swarming behavior with a shortened lag period (Figure 3A, 1B). All three strains swarmed at the same rate ($p = 1.24e^{-1}$) but swarming initiated at different times.

We then introduced an inducible copy of the *swrA* gene at an ectopic locus into our *yozG* mutant and tested the swarming phenotype. Again, overexpression of *swrA* restored swarming and the complementation swarmed at the same rate as wild type ($p = 1.95e^{-1}$) but there was still a lag period prior to the initiation of swarming, similar to wild type (Figure 3B, 1B).

Overexpression of yozG results in a reduction in growth rate

In addition to swarming, wild type *B. subtilis* can form robust floating-pellicle biofilms at liquid-air interfaces(25–28). Swarming and biofilm formation are opposing processes—mutations that enhance swarming often decrease biofilm formation and visa versa (26, 29). To test if there was a biofilm phenotype in our *yozG* strains we performed pellicle assays on both our *yozG* mutant and overexpression strains. Whereas the *yozG* mutant formed biofilms identical to wild type, overexpression of *yozG* resulted in a delay in pellicle formation and the production of a thinner, less textured biofilm, but only in the presence of inducer (Figure 4).

It is possible that the reduction in biofilm formation was caused by a reduction in growth rate. To determine if overexpression of *yozG* reduced growth, we measure the growth rate of cells in liquid culture. Overexpression of *yozG*

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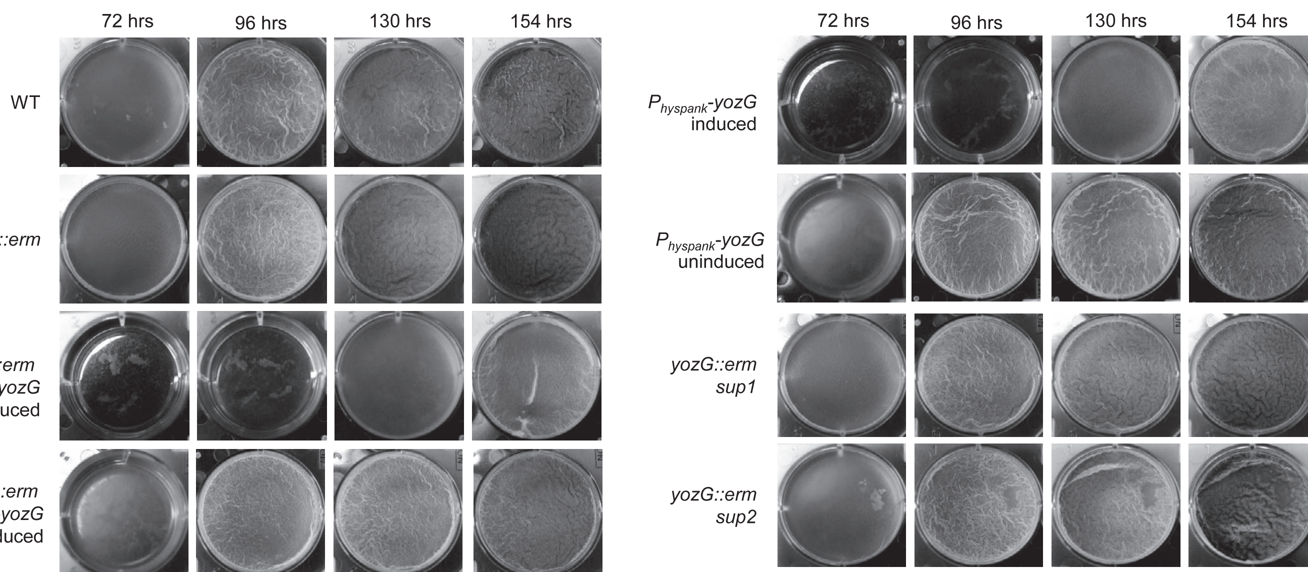
Figure 4. Overexpression of *yozG* results in delayed pellicle formation. Strains were inoculated into MSgg biofilm media and incubated at room temperature for 1 week. Pellicles are representative of triplicate experiments.



resulted in a reduced growth rate compared to wild type (Figure 5A, significant interaction effect, $p=1.62e^{-17}$). The growth defect was only apparent when inducer was added to stimulate overexpression of *yozG* (Figure 5B, $p = 4.02e^{-1}$). We infer that the reduction in biofilm formation is due to a reduction in growth rate when *yozG* is overexpressed.

This is consistent with the observation that the thickness of cell growth in our swarm expansion assay was reduced when *yozG* was overexpressed (Figure 1B). These data also could explain the plateau in the the swarming phenotype when the *yozG* overexpression construct was used to complement the mutant (Figure 1A). Swarming is highly dependent on rapid growth and high cell density for raft formation (6, 7). The growth defect caused by overexpression of *yozG* may counter the restoration of swarming due to a reduced cell density that is unable to support the swarm once it has expanded a certain distance.

Spontaneous genetic suppressors can restore swarming to the yozG mutant

During the course of our swarm expansion assays, we noticed that on some of the *yozG* mutant plates, flares of cells would swarm away from the central colony. Such flares generally represent suppressor mutations that restore swarming (8, 23, 30, 31). We isolated two such flares and performed swarm expansion assays using these cells. Both suppressor mutants had increased swarming compared to the *yozG* mutant, but neither swarmed identically to wild type (Figure 3C, $p = 1.0e^{-3}$). When tested for biofilm activity, both suppressor mutants formed robust biofilms identical to wild type and *yozG* mutant cells (Figure 4). We conclude that we have isolated genetic suppressor mutants of the *yozG* knockout that restore swarming motility.

Discussion

We have shown that the *yozG* gene is necessary for swarming motility in the undomesticated *B. subtilis* strain NCIB 3610. Knockout mutants of *yozG* failed to swarm, but the swarming phenotype could be restored by complementing the *yozG* gene at an ectopic locus in the chromosome. We have also shown that overexpression of *yozG* from an inducible promoter results in a slowed growth phenotype which causes a reduction in floating pellicle biofilm formation, and reduces the thickness of the colony after swarm expansion. Swarming could be restored to the *yozG* knockout by overexpressing the swarming regulator *swrA* or the flagella and chemotaxis operon *fla/che,* or by spontaneous suppressor mutations.

We were surprised that the swarming phenotype could be restored to our *yozG* complementation strain even in the absence of inducer. However, The $P_{hyspank}$ promoter used here is "leaky", and some level of expression is occurring in the absence of inducer. We infer from the growth phenotype that very low levels of YozG are needed in the cell, and that excess *yozG* expression has negative effects on growth.

SwrA controls entry into the swarming state by inducing expression of the *fla/che* operon (7–9, 25, 26). Levels of SwrA

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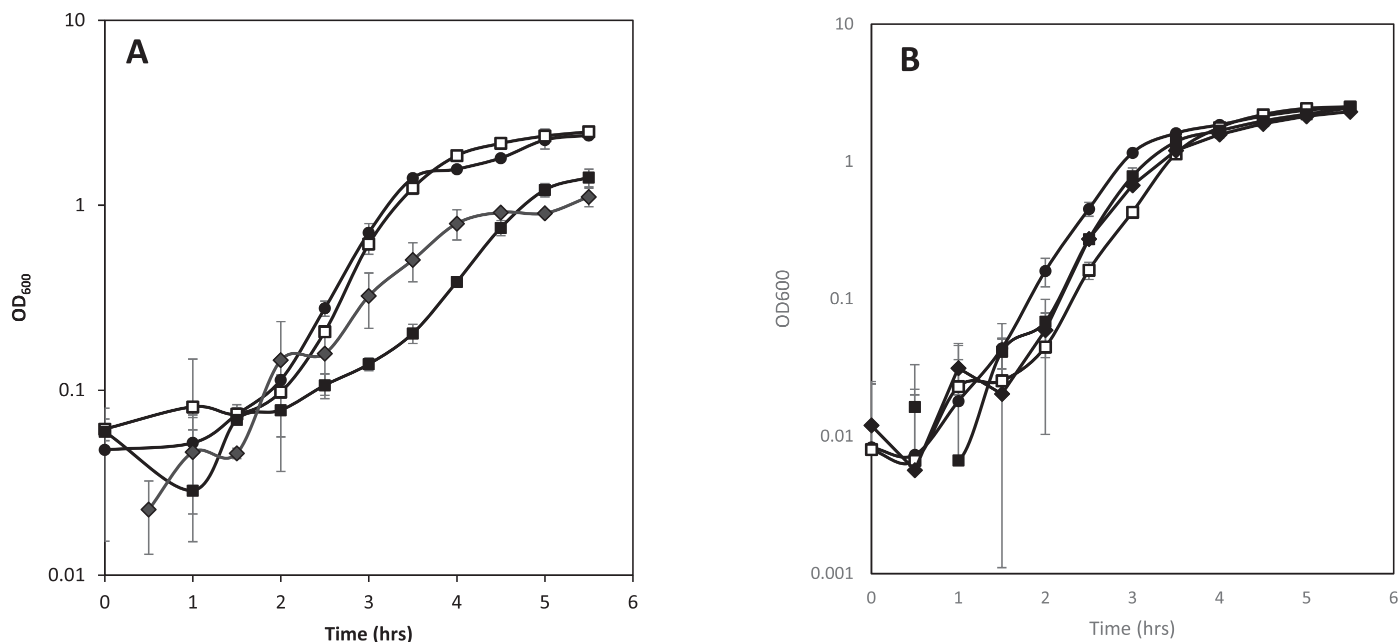
Figure 5. Overexpression of *yozG* reduced growth rate. Cells were inoculated into LB media with (A) 0.1mM IPTG or without (B) IPTG to a calculated starting OD_{600} of 0.01 and incubated at 37°C with shaking. OD measurements were taken every 30 minutes. Data is shown for wild type (DK1042, filled circles), *yozG* mutant (JS42, *yozG::erm*; empty squares), overexpression (JS43, *amyE::P*$_{hyspank}$*-yozG spec;* filled diamonds*)*, and complementation (JS51, *yozG::erm amyE::P*$_{hyspank}$*-yozG spec;* filled squares) strains. Lines represent the average of three replicates. Error bars represent twice the standard error of the mean.



in the cell are controlled by multiple factors. There is evidence that SwrA may positively regulate its own expression (10). *yozG* encodes a putative transcriptional regulator in the XRE family (16). The fact that overexpression of *swrA* was able to restore swarming to the *yozG* mutant suggests that YozG acts upstream of SwrA in the regulatory cascade. It is possible that YozG may act to increase transcription of *swrA*. SwrA protein levels are also controlled proteolytically by the protease LonA and the adapter SmiA (14, 15). YozG could inhibit *smiA* or *lonA* expression and therefore increase SwrA levels indirectly.

Master regulators in other swarming species, such as FlhDC in many gram-negative bacteria, are regulated at multiple levels by a variety of proteins (reviewed in (4)). YozG may represent another level of regulatory input into the control of the SwrA master regulator in *B. subtilis*. Further studies will be needed to determine if YozG influences *swrA* gene expression or SwrA protein levels *in vivo*. Identification of the site of the spontaneous suppressor mutations may also provide insight into the mechanism of *yozG* action.

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