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PHYSIOLOGY

Cell Aggregation and Aerobic Respiration Are Important for *Zymomonas mobilis* ZM4 Survival in an Aerobic Minimal Medium

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ABSTRACT *Zymomonas mobilis* produces ethanol from glucose near the theoretical maximum yield, making it a potential alternative to the yeast *Saccharomyces cerevisiae* for industrial ethanol production. A potentially useful industrial feature is the ability to form multicellular aggregates called flocs, which can settle quickly and exhibit higher resistance to harmful chemicals than single cells. While spontaneous floc-forming *Z. mobilis* mutants have been described, little is known about the natural conditions that induce *Z. mobilis* floc formation or about the genetic factors involved. Here we found that wild-type *Z. mobilis* forms flocs in response to aerobic growth conditions but only in a minimal medium. We identified a cellulose synthase gene cluster and a single diguanylate cyclase that are essential for both floc formation and survival in a minimal aerobic medium. We also found that NADH dehydrogenase 2, a key component of the aerobic respiratory chain, is important for survival in a minimal aerobic medium, providing a physiological role for this enzyme, which has previously been found to be disadvantageous in a rich aerobic medium. Supplementation of the minimal medium with vitamins also promoted survival but did not inhibit floc formation.

IMPORTANCE The bacterium *Zymomonas mobilis* is best known for its anaerobic fermentative lifestyle, in which it converts glucose into ethanol at a yield surpassing that of yeast. However, *Z. mobilis* also has an aerobic lifestyle, which has confounded researchers with its attributes of poor growth, accumulation of toxic acetic acid and acetaldehyde, and respiratory enzymes that are detrimental for aerobic growth. Here we show that a major *Z. mobilis* respiratory enzyme and the ability to form multicellular aggregates, called flocs, are important for survival, but only during aerobic growth in a medium containing a minimum set of nutrients required for growth. Supplements, such as vitamins or yeast extract, promote aerobic growth and, in some cases, inhibit floc formation. We propose that *Z. mobilis* likely requires aerobic respiration and floc formation in order to survive in natural environments that lack protective factors found in supplements such as yeast extract.

KEYWORDS NADH dehydrogenase, *Zymomonas mobilis*, biofilm, cellulose synthesis, cyclic di-GMP, diguanylate cyclase, ethanol, fermentation, flocculation, flocs

Zymomonas mobilis is a bacterium that can naturally produce ethanol from glucose near the theoretical maximum yield (1). Due to this high ethanol yield, *Z. mobilis* is often viewed as the bacterial counterpart to the yeast *Saccharomyces cerevisiae* (2, 3), although *Z. mobilis* has yet to be widely adopted for ethanol production on an industrial scale. Several physiological differences distinguish these two ethanol producers. For example, to metabolize sugar, *Z. mobilis* uses the low-ATP-yielding Entner-Doudoroff pathway, whereas *S. cerevisiae* uses the more energetically efficient Embden-Meyerhof-Parnas pathway. *Z. mobilis* can also use inexpensive N_2 gas as the sole

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nitrogen source without compromising its high ethanol yield (4), whereas yeast, like all eukaryotes, cannot use N_2. These two ethanol producers also differ in their abilities to tolerate inhibitory compounds in feedstock hydrolysates. For example, *S. cerevisiae* shows a greater tolerance of soy hydrolysate (5), and *Z. mobilis* shows a greater tolerance of drought-stressed switchgrass hydrolysate (6).

Z. mobilis also has an unusual aerobic lifestyle that sets it apart not only from *S. cerevisiae* but also from most aerobically respiring organisms. While capable of aerobic respiration, *Z. mobilis* employs an electron transfer chain that makes little, if any, contribution to ATP production (7). Instead, the net physiological effect of respiration appears to be harmful, since it competes with ethanol production for electrons, resulting in the accumulation of toxic acetaldehyde and acetic acid (7). Indeed, genetic or chemical disruption of respiration allows *Z. mobilis* to assume a purely fermentative lifestyle under aerobic conditions and actually improves growth trends (8–10).

Finally, another difference between *Z. mobilis* and *S. cerevisiae* is the tendency for *S. cerevisiae* to settle out of solution rapidly, in some cases aided by the formation of multicellular clusters called flocs (11). Flocculation can be advantageous for collecting cells or for immobilizing cells for use in continuous-flow bioreactors (11). The genetic factors behind *S. cerevisiae* flocculation are relatively well understood (12, 13), to such an extent that flocculation can be genetically manipulated (14). Flocs have also been observed in *Z. mobilis* and have been studied after enrichment for floc-forming mutants that arise spontaneously (15) or after chemical mutagenesis (16). Research on *Z. mobilis* flocs and biofilms (17) has thus far focused primarily on their utility in cell-recycle bioreactors (16) and their increased tolerance of inhibitory chemicals found in cellulosic hydrolysates (18). However, relatively little physiological and genetic characterization of *Z. mobilis* flocs has been performed beyond determining that cells within flocs are held together by an extracellular matrix containing cellulose (15, 19, 20). A gene cluster likely involved in cellulose synthesis was also identified recently (20).

Here we describe physiological conditions and genetic factors that are required for floc formation in wild-type *Z. mobilis* ZM4. We found that flocs form in response to aerobic conditions, but only when the organism is cultured in a minimal medium. We verified the involvement of a cellulose synthase gene cluster and additionally identified a single diguanylate cyclase (DGC) that is required for flocculation. We found that flocculation is required for cell viability in a minimal aerobic medium, suggesting a protective role against some aspect of aerobic metabolism. Furthermore, we found that NADH dehydrogenase 2 (NADH DH), a key enzyme in aerobic respiration, is important for survival in a minimal aerobic medium, thus revealing a physiological role for this enzyme, which has otherwise been found to be detrimental for growth in a rich aerobic medium (8–10).

RESULTS

***Z. mobilis* ZM4 forms flocs in a minimal aerobic medium.** Although best known for fermentative production of ethanol under anaerobic conditions, *Z. mobilis* is also capable of aerobic respiration (7). When we grew wild-type *Z. mobilis* ZM4 under aerobic conditions in *Zymomonas* minimal medium (ZYMM) with NH_4Cl as the sole nitrogen source, we observed that ZM4 formed flocs that were visible to the naked eye (Fig. 1A). Electron microscopy verified that the flocs were multicellular aggregates (Fig. 1B). Flocs were not observed when ZM4 was grown aerobically in the undefined peptone–yeast extract–glucose (PYG) medium (Fig. 1C). Thus, aerobic conditions alone are insufficient to stimulate floc formation. Flocs were also not observed when ZM4 was grown anaerobically in either ZYMM (Fig. 1D) or PYG medium (Fig. 1E). Thus, flocs do not form in response to some component of ZYMM. Rather, floc formation appears to be stimulated by a combination of aerobic conditions and growth in a minimal medium.

Flocculation can be quantified through an increase in turbidity upon dispersion. Flocs settle to the bottoms of test tubes but are easily observed after the tube is swirled (Fig. 1A). Flocs then settle again within seconds. To quantify the extent of

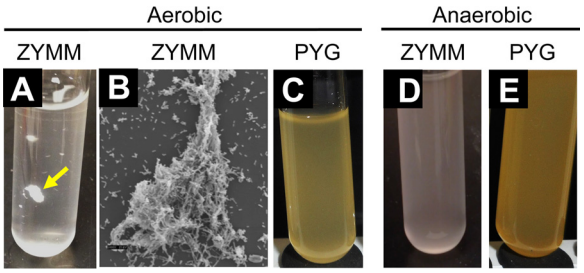

FIG 1 Flocs are observed only in a minimal aerobic medium. (A, C to E) Photographs of *Z. mobilis* ZM4 cultures in test tubes either with a minimal medium (ZYMM) or with the undefined, rich PYG medium. Media were swirled by hand before photographs were taken. The yellow arrow in panel A points to a macroscopic floc. (B) Scanning electron micrograph of a floc grown in aerobic ZYMM with NH_4Cl. Magnification, ×1,000 at 15 kV.

flocculation, we reasoned that the dispersion of flocs into individual cells should result in an increase in culture turbidity. Previous work by others on flocculant isolates of *Z. mobilis* determined that cellulose is a major component of the extracellular matrix; the addition of cellulase to floc-forming isolates dispersed flocs, whereas amylases, dextranase, pectinase, and hemicellulase did not (15, 19, 20). We verified that ZM4 flocs can be dispersed by cellulase, resulting in an increase in culture turbidity (Fig. 2). Protein and DNA can also be components of an extracellular matrix for some bacteria. However, we found that neither DNase nor proteinase K dispersed ZM4 flocs, nor did these enzymes enhance the dispersion of flocs when combined with cellulase (Fig. 2A). Adding cellulase and proteinase K together decreased the rate of turbidity increase, likely because the proteinase degraded the cellulase (Fig. 2A). Our findings support those of another group, which similarly found that protease did not disperse *Z. mobilis* flocs (20). Thus, the addition of cellulase alone can be used to quantify ZM4 flocculation by an increase in culture turbidity.

We also found that flocs could be mechanically dispersed by harsh vortexing. Vortexing at level 3 for 5 s, which we refer to as "gentle vortexing," briefly lifted flocs off the bottom of a test tube before they rapidly settled again. Vortexing with 10 pulses at maximum speed, which we refer to as "harsh vortexing," dispersed flocs and resulted in an increase in culture turbidity that was statistically similar to that observed by adding cellulase (Fig. 2B). We used these two approaches to assess floc formation in this study.

A cellulose synthase gene cluster and a single diguanylate cyclase are required for flocculation. Because the extracellular matrix of flocs contains cellulose, we examined the ZM4 genome sequence (21–23) for genes that could be responsible for cellulose synthesis. A gene cluster from ZMO1083 to ZMO1086 (*bcsABCZ*) was annotated as encoding a cellulose synthase complex (Fig. 3A) (24). ZMO1086, encoding BcsZ,

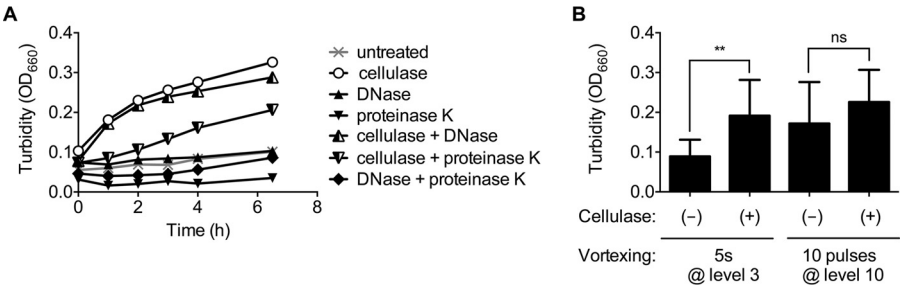

FIG 2 Flocs can be dispersed by cellulase or harsh vortexing. (A) Treatment of ZM4 flocs with cellulase, proteinase K, or DNase. Representative trends from one of two replicates are shown. (B) Effect of gentle (5 s at level 3) or harsh (10 pulses at level 10) vortexing on floc dispersal with (+) or without (–) a 3-h cellulase treatment. Error bars, standard deviations ($n = 4$). Asterisks indicate a significant difference (**, $P < 0.01$) by one-way analysis of variance with the Holm-Sidak posttest; ns, no significant difference.

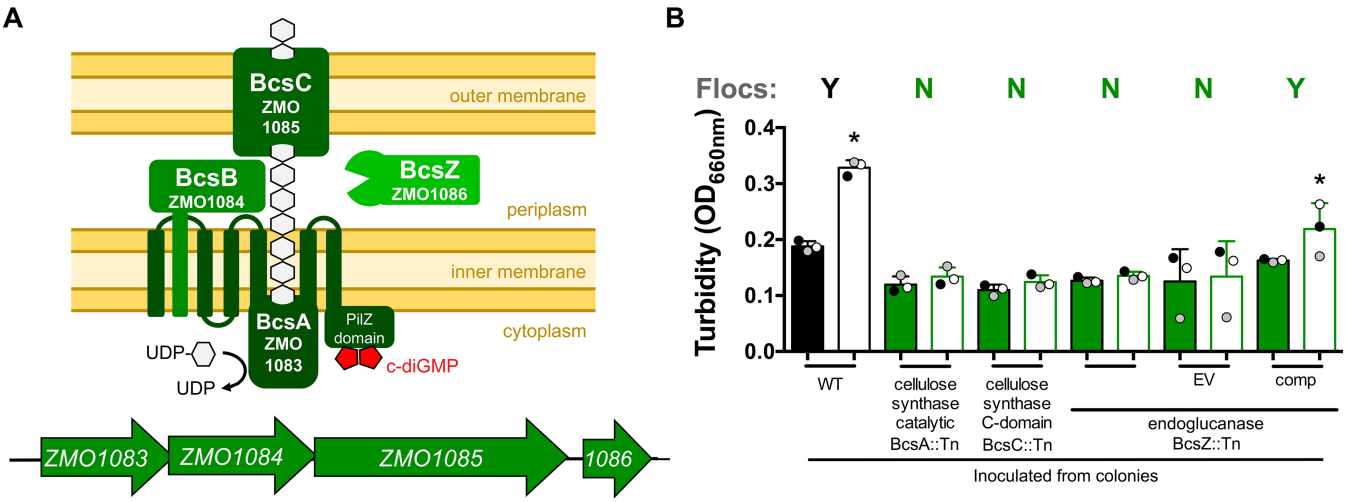

FIG 3 Floc formation requires cellulose synthase. (A) Cellulose synthase gene cluster (ZMO1083 to ZMO1086) (bottom) and a schematic of the resulting protein complex (top) based on references 20 and 24. (B) Turbidities of aerobic cultures grown from colonies in a minimal medium (ZYMM) before (filled bars) and after (open bars) cellulase treatment. Shaded circles represent the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed. WT, wild-type ZM4; EV, empty vector pSRKT; comp, complementation vector pSRKTc-BcsZ. Error bars, standard deviations. An asterisk indicates a significant difference from the value for the corresponding filled bar (*, $P < 0.1$) by one-way analysis of variance with Sidak's multiple-comparison posttest.

had been purified previously, and its endoglucanase activity had been characterized (25). More recently, another group found that genetic disruption of the BcsA catalytic subunit, encoded by ZMO1083, prevented floc formation in a constitutively flocculant *Z. mobilis* mutant (20). We tested three ZM4 mutants, each containing a transposon (Tn) insertion in a different gene in this cluster, for their abilities to form flocs in aerobic ZYMM. None of the mutants formed flocs in this minimal aerobic medium (Fig. 3B). We chose to complement the BcsZ::Tn mutant, since BcsZ is the terminal gene in the operon and thus is the least likely to have polar effects from the Tn insertion. The expression of *bcsZ* from its native promoter on a plasmid restored floc formation to the BcsZ::Tn mutant, whereas the mutant with an empty vector failed to form flocs (Fig. 3B). We concluded that this gene cluster is required for floc formation, most likely through the production of extracellular cellulose.

Clustering of cells into flocs or biofilms is commonly coordinated by the intracellular levels of cyclic di-GMP (c-di-GMP) (26). c-di-GMP is produced by DGCs, which typically have a GG(D/E)EF domain, and is degraded by phosphodiesterases (PDEs), which often have an EAL domain (27). The ZM4 genome has five genes annotated as having GG(D/E)EF and/or EAL domains (Fig. 4A). We examined five Tn mutants, one for each of the five genes, for flocculation in ZYMM. Only a Tn insertion in gene ZMO0919, encoding a predicted DGC (referred to below as DGC_{0919}), prevented floc formation (Fig. 4B). Floc formation was restored in the DGC_{0919}::Tn mutant by expressing ZMO0919 under the control of its native promoter from a plasmid, whereas an empty vector did not restore flocculation (Fig. 4B). Based on these observations, we conclude that DGC_{0919} is required for floc formation, likely through the production of c-di-GMP.

Mutants incapable of floc formation have low viability in a minimal aerobic medium. Mutants that did not form flocs in the minimal aerobic medium also had final optical densities (ODs) lower than those of dispersed ZM4 cultures (Fig. 3B and 4B). This discrepancy in final OD values was exaggerated when mutant cultures were transferred to fresh ZYMM (Fig. 5A). These observations suggested that an inability to form flocs resulted in cell death, preventing culture growth after transfer to fresh medium. Indeed, the viable-cell counts of the BcsZ::Tn mutant were below the detection limit (~1,000 CFU/ml) after it was transferred and incubated in fresh ZYMM, unless *bcsZ* was expressed from a plasmid (Fig. 5B). In a separate experiment, we also plated the BcsZ::Tn mutant for CFU immediately after vortexing flocs in cultures that had grown from colonies in aerobic ZYMM. CFU developed only for ZM4 and the complemented

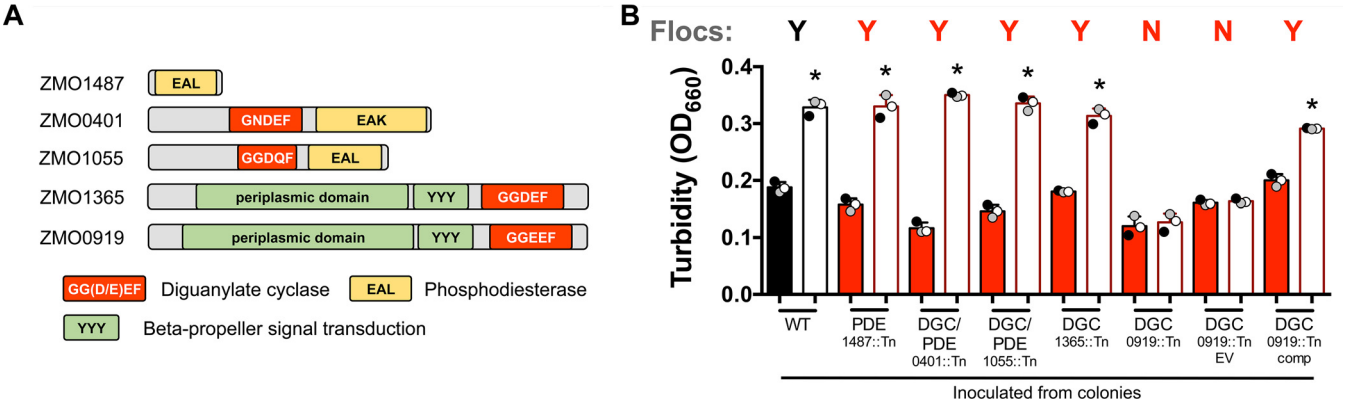

FIG 4 Floc formation requires cellulose synthase and a specific diguanylate cyclase. (A) Domains of ZM4 proteins with predicted diguanylate cyclase GG(D/E)EF domains (red) or phosphodiesterase EAL domains (yellow). (B) Turbidities of aerobic cultures grown from colonies in a minimal medium (ZYMM) before (filled bars) and after (open bars) cellulase treatment. Shaded circles indicate the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed. WT, wild-type ZM4; EV, empty vector pSRKT; comp, complementation vector pSRKTc-DGC0919. Error bars, standard deviations. An asterisk indicates a significant difference from the value for the corresponding filled bar (*, $P < 0.1$) by one-way analysis of variance with Sidak's multiple-comparison posttest.

BcsZ::Tn strain, not for the BcsZ::Tn mutant with or without an empty vector (data not shown; detection limit, 100 CFU/ml). Thus, the BcsZ::Tn mutant was not viable even before transfer to fresh aerobic medium. Turbid growth of the BcsZ::Tn mutant was indistinguishable from that of ZM4 when the strains were grown from colonies in anaerobic ZYMM or in aerobic PYG medium (Fig. 5C). Thus, the poor growth of the BcsZ::Tn mutant in aerobic ZYMM was not due to a general growth defect.

Respiratory NADH dehydrogenase is important for survival in a minimal aerobic medium. We questioned whether floc formation was stimulated by the aerobic environment or by some aspect of aerobic respiration. NADH DH, encoded by ZMO1113 (*ndh*), is the primary enzyme by which electrons enter the aerobic electron transfer chain in *Z. mobilis* (8–10). When cultured in a rich aerobic medium, *Z. mobilis* NADH DH mutants have been reported to behave as if they were under anaerobic conditions, exhibiting improved growth and a high ethanol yield (8–10) instead of the high levels of acetaldehyde and acetic acid typical of respiring *Z. mobilis* (7). We verified that three different NADH DH Tn mutants (Ndh::Tn) exhibited similar trends of improved growth and higher ethanol yields in aerobic PYG medium (Fig. 6). These trends could be attributed to the loss of *ndh*, since *ndh* expression from a plasmid in an

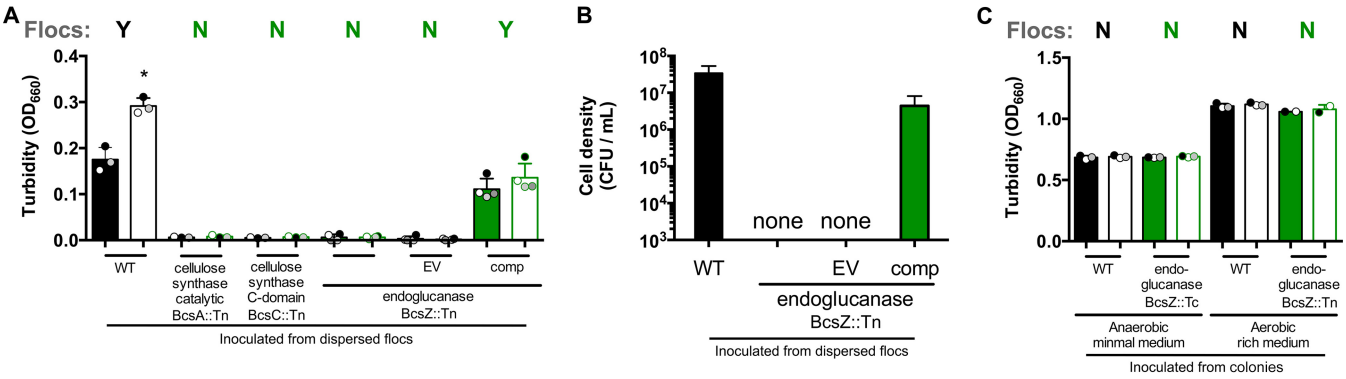

FIG 5 Cellulose synthase genes are required for survival in a minimal aerobic medium. (A) Cultures in a minimal aerobic medium, ZYMM, inoculated from harshly vortexed cultures under the conditions used for Fig. 3. (B) CFU from cultures used for panel A plated onto PYG agar. "None" indicates that no colonies were observed in 10 μl of a 10^{-1} dilution. (C) Cultures in anaerobic ZYMM or aerobic PYG medium inoculated from colonies. (A and C) Turbidities of cultures after gentle vortexing (5 s, level 3) (filled bars) and after dispersion of flocs by harsh vortexing (10 pulses, level 10) (open bars). Shaded circles indicate the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed. An asterisk indicates a significant difference from the corresponding filled bar (*, $P < 0.1$) by one-way analysis of variance with Sidak's multiple-comparison posttest. WT, wild-type ZM4; EV, empty vector pSRKTc; comp, complementation vector pSRKTc-BcsZ. Error bars, standard deviations.

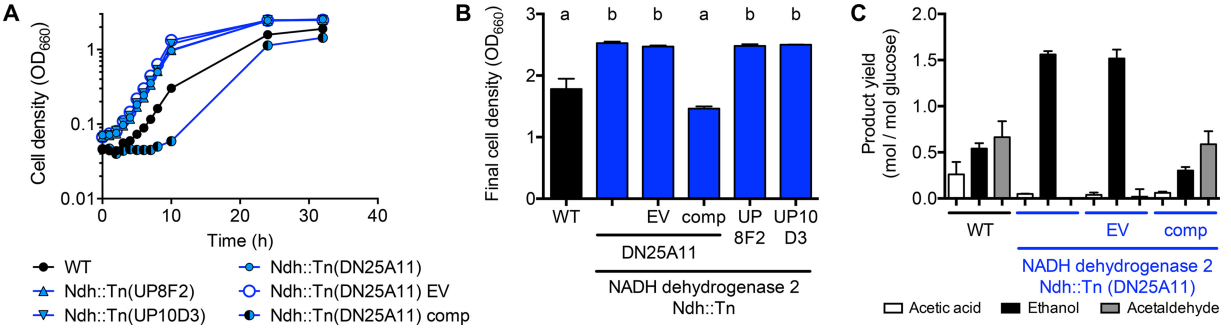

FIG 6 NADH DH mutants show improved growth trends and a higher ethanol yield in rich PYG medium. Shown are representative growth curves (A), final OD values (B), and major fermentation product yields (C) for wild-type ZM4 (WT) and NADH DH mutants (Ndh::Tn) in PYG medium. EV, empty vector pSRKTc; comp, complementation vector pSRKTc-Ndh. (B) Bars with different letters are significantly different ($P < 0.05$) as determined by one-way analysis of variance with Sidak's multiple-comparison posttest. Error bars, standard deviations ($n = 2$). Trends similar to those shown in panel A were observed for at least one other biological replicate.

Ndh::Tn mutant resulted in a lower final OD and an accumulation of acetic acid and acetaldehyde in an aerobic PYG medium, findings similar to those for ZM4 (Fig. 6).

In contrast to what we observed in PYG medium, the Ndh::Tn mutants formed visible flocs in aerobic ZYMM, though to a lesser extent than ZM4 cultures, such that a change in OD was often negligible after dispersion (Fig. 7A). Thus, floc formation is not solely a response to some aspect of NADH DH activity but is likely influenced by it. Also in contrast to the results in aerobic PYG medium, Ndh::Tn cultures grew less than ZM4 cultures in aerobic ZYMM. This trend of poor growth was exaggerated upon the transfer of dispersed Ndh::Tn mutant cultures to fresh medium (Fig. 7B and C). The expression of *ndh* from a vector improved the survival of an Ndh::Tn mutant, whereas an empty vector did not (Fig. 7). Thus, we conclude that while NADH DH activity is dispensable and perhaps even detrimental in a rich aerobic medium such as PYG medium, it is important for survival in a minimal aerobic medium.

The poor viability of NADH DH mutants in aerobic ZYMM rules out several possible explanations for the low viability of the DGC_{0919}::Tn and BcsZ::Tn flocculation mutants. First, since NADH DH mutants produce little acetic acid, the death of flocculation mutants was not likely due to acidification from acetic acid accumulation. In further support of this notion, supplementation of aerobic ZYMM with 100 mM morpholinepropanesulfonic acid (MOPS), pH 7, did not stimulate the growth of the DGC_{0919}::Tn or BcsZ::Tn mutant, despite partially alleviating the pH drop (Fig. 8). Second, because

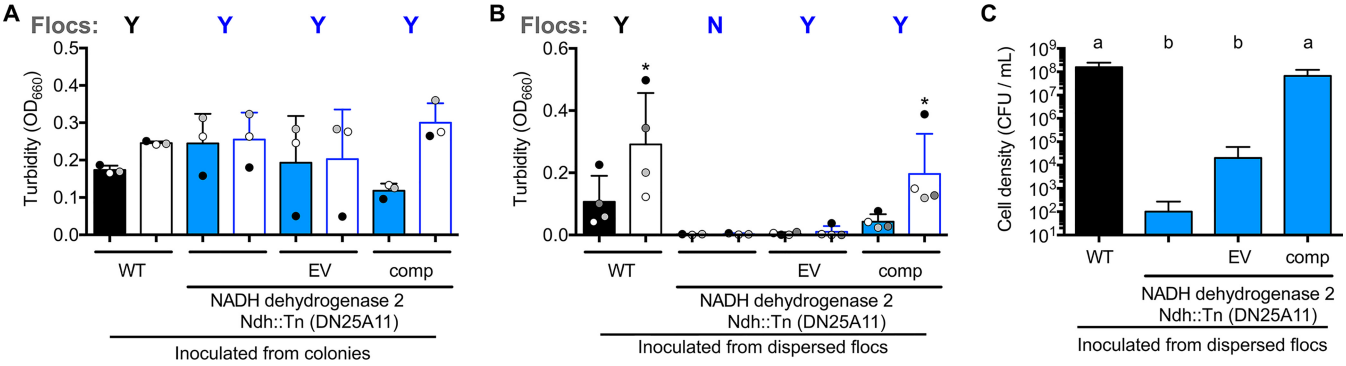

FIG 7 NADH DH is important for survival in a minimal aerobic medium. (A and B) Turbidities of cultures grown in a minimal aerobic medium (ZYMM) after gentle vortexing (5 s, level 3) (filled bars) and after dispersion of flocs by harsh vortexing (10 pulses, level 10) (open bars). Cultures were inoculated from colonies (A) or from harshly vortexed cultures from panel A (B). Shaded circles indicate the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed. An asterisk indicates a significant difference from the corresponding filled bar (*, $P < 0.1$) by one-way analysis of variance with Sidak's multiple-comparison posttest. (C) CFU from panel B cultures that were plated onto PYG agar. Bars with different letters are significantly different ($P < 0.05$) as determined by one-way analysis of variance with Sidak's multiple-comparison posttest. Error bars, standard deviations. EV, empty vector pSRKTc; comp, complementation vector pSRKTc-Ndh.

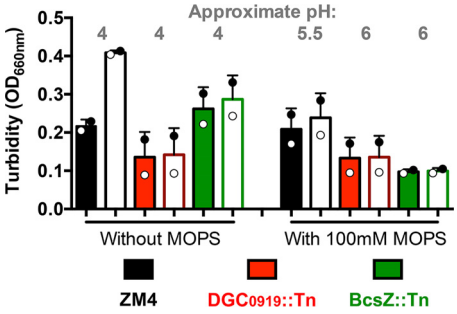

FIG 8 The addition of MOPS buffer does not promote growth in a minimal aerobic medium. Shown are the turbidities of cultures grown from colonies in a minimal aerobic medium (ZYMM) without or with 100 mM MOPS after gentle vortexing (5 s, level 3) (filled bars) and after harsh vortexing (10 pulses, level 10) to disperse any flocs (open bars). Shaded circles indicate the same culture pre- and postdispersion.

NADH DH mutants produce little acetaldehyde, the death of flocculation mutants was not likely due to acetaldehyde accumulation alone. We also tested whether acetaldehyde could be a factor that stimulates floc formation by adding acetaldehyde to cultures growing in anaerobic ZYMM, conditions under which acetaldehyde is normally not produced. The addition of acetaldehyde to anaerobic ZYMM in sealed test tubes resulted in a lower final OD but did not induce floc formation (Fig. 9).

Vitamins as possible protective factors in yeast extract. Whereas ZM4 always formed flocs in aerobic ZYMM, we did not observe flocs when ZM4 was grown aerobically in PYG medium (Fig. 1). Many other studies of aerobically grown *Z. mobilis* used a medium with 0.5% yeast extract as the only undefined supplement (8, 9, 28–32). We therefore tested whether the addition of 0.5% yeast extract to ZYMM would improve growth and/or prevent floc formation. The addition of yeast extract averted the toxic effects of aerobic growth for all strains tested (Fig. 10A). However, the effect on floc formation in ZM4 cultures was variable; flocs were observed in ZM4 cultures inoculated from colonies, although the cultures were mostly turbid, as seen from the negligible change in OD after harsh vortexing (Fig. 10A). In subsequent cultures inoculated from these harshly vortexed cultures, no flocs were visible to the eye (Fig. 10B). Similar effects on floc formation were observed for an Ndh::Tn mutant (Fig. 10A and B).

We then screened several factors that could possibly explain why supplements such as yeast extract might stimulate aerobic growth. Specifically, we tested amino acids with antioxidant properties (33–37), mineral availability, and vitamins. The amino acid supplement was based on the expected concentrations of arginine, aspartate, glutamate, methionine, and proline in yeast extract (38). The mixed amino acid supplement

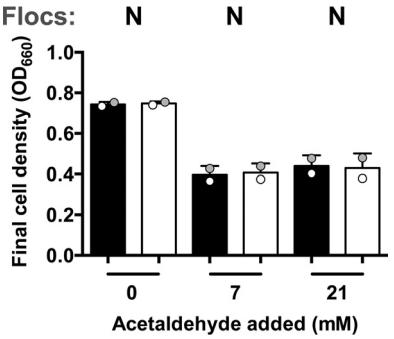

FIG 9 Acetaldehyde does not induce floc formation under anaerobic conditions. Shown are the turbidities of ZM4 cultures grown in a minimal anaerobic medium (ZYMM) with the indicated amount of acetaldehyde after gentle vortexing (5 s, level 3) (filled bars) and after harsh vortexing (10 pulses, level 10) to disperse any flocs (open bars). Shaded circles indicate the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed.

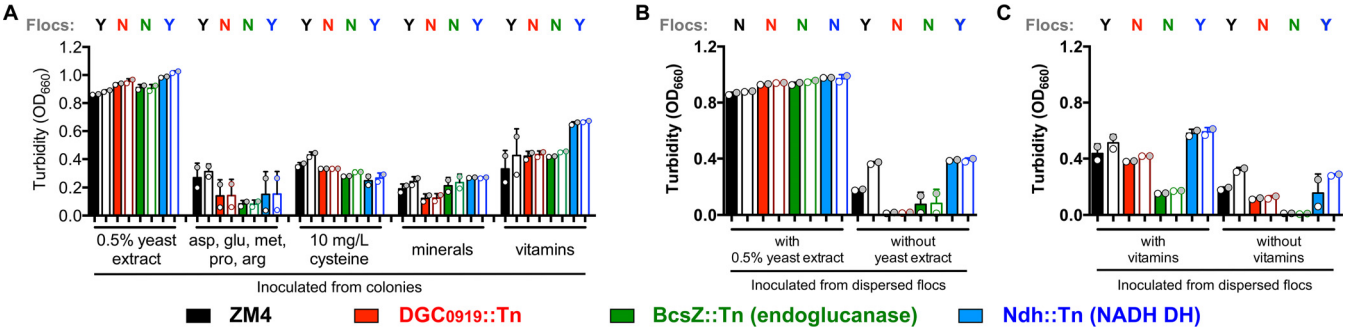

FIG 10 Yeast extract and vitamins promote the growth of oxygen-sensitive mutants. Shown are the turbidities of cultures grown in a minimal aerobic medium (ZYMM) with the indicated supplement after gentle vortexing (5 s, level 3) (filled bars) and after the dispersion of flocs by harsh vortexing (10 pulses, level 10) (open bars). Cultures were inoculated from colonies (A) or from harshly vortexed cultures from panel A (B and C). Shaded circles indicate the same culture pre- and postdispersion. Y, visual observation of flocs; N, no flocs observed.

did not improve growth trends, nor did increasing the mineral concentration 10-fold over what is normally provided in ZYMM (Fig. 10A). Cysteine is a potent antioxidant and is commonly used to scavenge O_2 in anaerobic media (37). Although it is not listed as a component of yeast extract, we tested a cysteine supplement based on the expected concentration of oxidized cystine in yeast extract (38). The cysteine supplement appeared to have a mild stimulatory effect on aerobic growth in ZYMM, more than the amino acid mixture or the higher concentration of minerals (Fig. 10A). Yeast extract is also rich in B vitamins, some of which can act as redox mediators (39). We therefore tested the addition of a readily available vitamin mixture (40) with B vitamins within the range typical for yeast extract (39). The vitamin supplement stimulated the aerobic growth of all strains, with the Ndh::Tn mutant notably showing a higher final OD, similar to what was observed in aerobic PYG medium (compare Fig. 10A with Fig. 6B).

To determine whether vitamins improved the survival of oxygen-sensitive mutants, we then transferred a 1% inoculum of stationary-phase-cultures to fresh identical media with and without the supplements. While all strains grew to higher final ODs with vitamins than without vitamins, the two flocculation mutants did not grow as much as when inoculated from colonies (compare Fig. 10A and C). Thus, while the vitamin supplement promoted the aerobic growth of all strains, it did not appear to completely protect against the toxic aspects of aerobic growth.

We attempted to gain further insight into the conditions that might protect *Z. mobilis* during aerobic growth by examining a chemogenomic profiling database in which the fitness effects from Tn disruptions of nearly every *Z. mobilis* ZM4 gene were tested across hundreds of conditions, including aerobic and anaerobic conditions with and without undefined supplements (41). However, none of the mutants examined in our study showed a significant growth defect during aerobic growth in a defined medium in the chemogenomic profiling database (41) (Fig. 11). In fact, the cellulose synthase gene cluster (BcsABCZ) Tn mutants were reported to generally have positive fitness values across all conditions (41). The database also did not agree with predictions from our study and others (8–10) that an interruption of *ndh*, encoding NADH DH, would result in a fitness advantage in rich aerobic media (41). We speculate that these discrepancies are due to differences in the way the experiments were performed. For example, the chemogenomic profiling was carried out in 10-ml volumes (41), which might have had more-limited aeration than the 5-ml volumes we used. Also, the defined medium used in the chemogenomic profiling contained four different B vitamins (41), which we showed can limit the toxic effects of aerobic growth (Fig. 10). Another possibility is that growing the entire library of mutants in the same test tube for chemogenomic profiling could have led to cross-complementation, rescuing mutants that would otherwise be subject to the toxic effects of aerobic growth if grown as a clonal population.

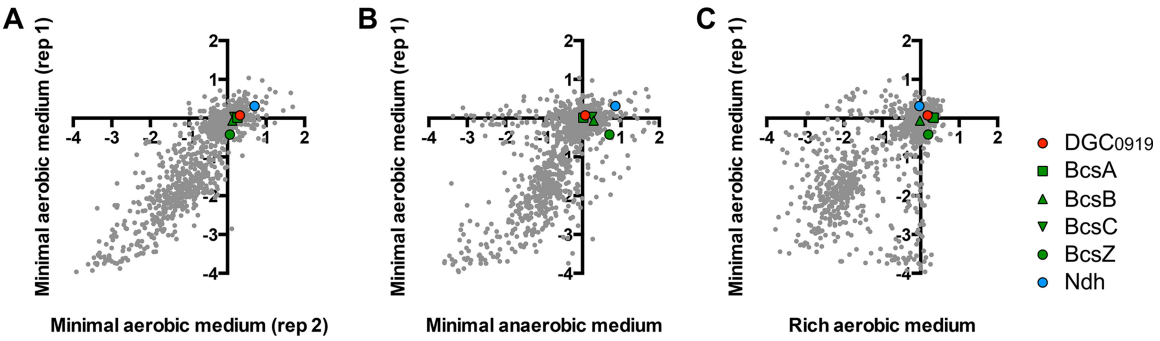

FIG 11 Comparison of gene fitness values from a chemogenomic profiling study (41). Values along the *x* and *y* axes are gene fitness values for the growth conditions indicated. Gene fitness values reflect the change in population frequency for a given Tn mutant from the time of inoculation until the end of the experiment (6 to 8 generations). Negative values indicate that a Tn insertion lowered strain fitness. Positive values indicate that a Tn insertion increased strain fitness. More information on the conditions and calculations used to determine gene fitness values is available elsewhere (41, 44). Each gray dot represents the gene fitness value for a given ZM4 gene. Graphs compare gene fitness values in a minimal aerobic medium (rep 1) with those for a replicate under an identical growth condition (control; rep 2) (A) or with those in a minimal anaerobic medium (B) or a rich aerobic medium (C).

DISCUSSION

We have demonstrated that the ability to form flocs and the aerobic respiration enzyme NADH DH are important for the survival of *Z. mobilis* ZM4 in a defined aerobic medium. Floc formation and respiration might work in concert; by concentrating respiratory activities, the community of cells in a floc might create a protective anaerobic microenvironment. The importance of cell clustering might also help explain why flocculation mutants grew from colonies but did not grow when transferred to fresh medium (Fig. 5 and 10). However, in opposition to this notion of synergistic activities, it is not obvious why starting respiration-deficient NADH DH mutant cultures from colonies would promote growth (Fig. 7). Rather, the formation of flocs and initial growth by an NADH DH mutant suggest that flocs might have protective properties independent of respiration. Such protection could be similar to that afforded by the exopolysaccharide secreted by *Leuconostoc mesenteroides*, which has been shown to protect cells from reactive oxygen by lowering the dissolved O_2 concentration, although the biochemical mechanism by which this occurs remains unknown (42). One possible factor contributing to the poor growth of the NADH DH mutant upon transfer could be that cultures were transferred after turbidity had leveled off, indicating a cessation of growth. There is evidence that NADH DH activity is important for survival in starved cultures that are periodically fed glucose (28). Thus, a disadvantage under starvation conditions could contribute to an explanation for the poor growth of NADH DH mutants upon transfer.

Undefined supplements promoted the growth of all strains tested and, in some cases, inhibited floc formation (Fig. 1 and 10). We determined that mineral availability, antioxidant amino acids, and vitamins by themselves cannot fully explain why supplements such as yeast extract play a protective role (Fig. 10). One possibility is that yeast extract contributes to a reduced environment, since yeast extract has been shown to lower the oxidation-reduction potential of media (39); components in yeast extract might help to keep critical cell components in a reduced state during aerobic growth. Redox-active B vitamins in yeast extract might contribute to this role (39, 43) and might also help explain why the addition of vitamins alone promoted aerobic growth in ZYMM (Fig. 10). Another possibility is that yeast extract and vitamins provide a critical nutrient that *Z. mobilis* has difficulty synthesizing in an aerobic environment, perhaps due to an O_2-sensitive enzyme.

The use of yeast extract in studies of *Z. mobilis* aerobic respiration has also led to observations by others that NADH DH activity, and respiration in general, are dispensable and even come with a fitness cost during growth (8–10, 30). These observations raise questions as to how genes that seemingly come with a fitness cost, such as the

TABLE 1 Strains, plasmids, and primers

Strain, plasmid, or primer	Relevant characteristics[a]	Source or reference
Strains[b]		
ATCC 31821 (ZM4)	Wild type	44
UP8_C10 (BcsA::Tn)	ZMO1083::Tn*5* Kn^r (cellulose synthase catalytic subunit BcsA)	44
DN23_B1 (BcsC::Tn)	ZMO1085::Tn*5* Kn^r (cellulose synthase C-domain protein BcsC)	44
UP4_H7 (BcsZ::Tn)	ZMO1086::Tn*5* Kn^r (endoglucanase BcsZ)	44
DN8_H11 (DGC/PDE$_{0401}$::Tn)	ZMO0401::Tn*5* Kn^r (DGC/PDE)	44
DN12_H6 (DGC$_{0919}$::Tn)	ZMO0919::Tn*5* Kn^r (DGC)	44
DN36_C3 (DGC/PDE$_{1055}$::Tn)	ZMO1055::Tn*5* Kn^r (DGC/PDE)	44
DN18_A11 (DGC$_{1365}$::Tn)	ZMO1365::Tn*5* Kn^r (DGC)	44
DN9_H1 (PDE$_{1487}$::Tn)	ZMO1487::Tn*5* Kn^r (PDE)	44
DN25_A11 (Ndh::Tn)	ZMO1113::Tn*5* Kn^r (NADH dehydrogenase 2)	44
UP8_F2 (Ndh::Tn)	ZMO1113::Tn*5* Kn^r (NADH dehydrogenase 2)	44
UP10_D3 (Ndh::Tn)	ZMO1113::Tn*5* Kn^r (NADH dehydrogenase 2)	44
Plasmids		
pSRKTc	Empty vector; Tc^r	47
pSRKTc-DGC0919	DGC$_{0919}$::Tn complementation vector; Tc^r	This study
pSRKTc-BcsZ	BcsZ::Tn complementation vector; Tc^r	This study
pSRKTc-Ndh	Ndh::Tn complementation vector; Tc^r	This study
Primers		
pSRKTc-0919_For_SacI	ACTAGAGCTCGGCTGCTTTTCGTATATGC	This study
pSRKTc-0919_Rev_KpnI	GAGGGTACCGGCGATAATGCCCAAAATTC	This study
pSRKTc-BcsZ_For_SacI	AAAAGAGCTCGAAGCCATATTTTCTTTAATTATGAAAGAT	This study
pSRKTc-BcsZ_Rev_KpnI	AAAGGTACCCCTTGCCCAATCCTCATAAAAAAATGATAG	This study
pSRKTc-Ndh_For_KpnI	AAAGGTACCGACGGGAAAAGAAGCCAAAGGTCA	This study
pSRKTc-Ndh_Rev_SacI	AAAGAGCTCCCTTTGGTCTTTCTTTTAAAAAGGCTCATTACT	This study

[a]For strains, the genotype (disrupted gene product) is given; for primers, the sequence (5′ → 3′) is shown, with restriction sites underlined. Kn^r, kanamycin resistance cassette; Tc^r, tetracycline resistance cassette; DGC, diguanylate cyclase; PDE, phosphodiesterase. *Z. mobilis* gene numbers are given as "ZMO" followed by four digits (21).

[b]For each strain, the designation used in the text is given in parentheses.

gene encoding NADH DH, have been maintained in *Z. mobilis*. Our observations showing that NADH DH is important for survival in a defined aerobic medium (Fig. 7) suggest that aerobic respiration is important for survival in natural environments that are deficient in protective factors such as those found in yeast extract.

The exact mechanism of toxicity and the signal for flocculation under aerobic conditions remain elusive. Nonetheless, we have uncovered general conditions and key genetic factors responsible for flocculation and survival. This knowledge could be used to control flocculation and potentially to promote survival in response to stress conditions in industrial hydrolysates.

MATERIALS AND METHODS

Strains and growth conditions. All strains are described in Table 1. *Zymomonas mobilis* ZM4 (ATCC 31821) and all Tn mutants were provided by J. M. Skerker and A. P. Arkin, University of California at Berkeley (44). When only one Tn mutant was used, we selected the mutant with the insertion site closest to the N terminus. Exact insertion site locations are listed elsewhere (44). Transposon insertions were verified by PCR and the sequencing of PCR products. Strains were streaked from frozen 25% glycerol stocks preserved at −80°C onto PYG agar (2% peptone, 1% yeast extract, 2% glucose, 1.5% agar) with antibiotics as appropriate. For growth experiments, colonies were then inoculated to either 5 ml of an aerobic liquid medium or 10 ml of an anaerobic liquid medium and were incubated at 30°C with shaking at 225 rpm. ZYMM with 50 mM glucose, 100 nM calcium pantothenate, and 10 mM NH_4Cl was used as the minimal medium (4). Where indicated, cultures were also supplemented with a mixture of amino acids at final concentrations of 0.1 mg/ml for aspartate, arginine, methionine, and proline, and with 0.6 mg/ml potassium glutamate. The cysteine-HCl supplement was added to a final concentration of 0.01 mg/ml. The mineral supplement contained 10-fold more of the mineral supplement typically added to ZYMM (4). The vitamin supplement was added from a 100× stock solution described elsewhere (40). Unless stated otherwise, aerobic test tubes had loose-fitting caps to allow for air exchange. For anaerobic conditions, the medium was bubbled with N_2 gas, and the tubes were then sealed with rubber stoppers (Geo-Microbial Technologies, Ochelata, OK) and aluminum crimps. *Escherichia coli* strains used for cloning were grown in LB broth or on LB agar. Where appropriate, tetracycline was used at 5 μg/ml for *Z. mobilis* and at 15 μg/ml for *E. coli*, and kanamycin was used at 100 μg/ml for *Z. mobilis*.

Construction of complementation vectors. All plasmids and primers are described in Table 1. All enzymes and competent cells were used according to the manufacturer's instructions. Primers were designed

to amplify entire genes and promoter regions as predicted using the BPROM algorithm (Softberry, Inc.) (45) and to introduce flanking ScaI and KpnI restriction sites. PCR products were then digested and ligated into pSRKTc, which had been cut with the same enzymes. The ligation reaction product was transformed into *E. coli* NEB10β (New England BioLabs) and was plated onto LB agar with tetracycline. Transformants were screened by PCR and verified by sequencing. Purified plasmids were then transformed into electrocompetent cells of the appropriate *Z. mobilis* strain as described elsewhere (46).

Cellulase and mechanical dispersion of flocs. All vortexing levels are those for a VWR Mini Vortexer. Predispersion turbidity was assessed after cultures were vortexed at level 3 for 5 s. To enzymatically disperse flocs, 50 μl of *Aspergillus niger* cellulase (1%, wt/vol; MP Biomedicals) in 0.1 M morpholineethanesulfonic acid (MES) buffer, pH 3.5, was added per 1 ml of culture, and cultures were incubated overnight at 30°C. Treated cultures were then vortexed for 5 s at level 6 prior to measurement of the optical density at 660 nm (OD_{660}). To mechanically disperse flocs, cultures were vortexed with 10 pulses at level 10. Dispersion was performed after the turbid fraction had reached a maximum OD_{660} value, typically within 6 days, though sometimes longer for slow-growing strains harboring plasmids. Culture turbidity was assayed by reading the OD_{660} using a Genesys 20 visible spectrophotometer (Thermo Fisher, Pittsburgh, PA).

Enzymatic characterization of the extracellular matrix. Enzymes were added to flocs grown in aerobic ZYMM, and cultures were then added to each test tube and were incubated at 30°C with shaking at 225 rpm. Cultures were periodically resuspended by vortexing at level 3 for 5 s, and optical density readings were taken. Stock solutions of enzymes were prepared in water, except for cellulase, which was prepared in 0.1 M MES, pH 3.5. Final concentrations were as follows: DNase, 20 μg/ml; proteinase K, 800 μg/ml; cellulase, 100 μg/ml.

Scanning electron microscopy. Flocculent cultures were added to 0.1% poly-L-lysine-treated glass coverslips and were incubated at room temperature for 5 min. Cells were then fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS) and were incubated at 4°C for 1 h. The fixative was removed by three washes with PBS at 4°C. The sample was dehydrated using a graded ethanol series (30, 50, 70, 90, and 95%), at 4°C for 5 min at each concentration, followed by three changes of 100% ethanol at room temperature for 5 min each. The sample was thin-sectioned and critical-point dried using CO_2 in a Balzers critical-point dryer, model CPD 030. The dried sample was then placed on an aluminum stub, sputter-coated with gold-palladium (60:40), and imaged using a JEOL JSM-5800 scanning electron microscope at the Indiana University Electron Microscopy Center.

Statistics. All statistical analyses were performed using Prism 6.0h (GraphPad Software, Inc.)

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