

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 yeast 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. While spontaneous floc-forming *Z. mobilis* mutants have been described, little is known about the natural conditions that induce *Z. mobilis* floc formation and 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 an aerobic minimal medium. We also found that NADH dehydrogenase 2, a key component of the aerobic respiratory chain, is important for survival in an aerobic minimal medium, providing a physiological role for this enzyme which has previously been found to be disadvantageous in aerobic rich media. 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 that surpasses that of yeast. However, *Z. mobilis* also has an aerobic lifestyle which has confounded researchers with 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 like 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 to survive in natural environments that lack protective factors found in supplements like yeast extract.

Introduction

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

45 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
50 more energetically efficient Embden-Myerhof-Parnas pathway. *Z. mobilis* can also use
inexpensive N₂ gas as the sole nitrogen source without compromising the high ethanol yield (4)
whereas yeast, like all eukaryotes, cannot use N₂. These two ethanol producers also differ in
their ability 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).

55 *Z. mobilis* also has an unusual aerobic lifestyle that sets it apart not only from *S. cerevisiae* but
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, as it competes against ethanol
60 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*
65 to rapidly settle out of solution, 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 the extent that flocculation can be genetically
manipulated (14). Flocs have also been observed in *Z. mobilis* and have been studied after
70 enriching for floc-forming mutants that arise spontaneously (15) or after chemical
mutagenesis (16). Research on *Z. mobilis* flocs, and biofilms (17), has thus far primarily focused
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
75 within flocs are held together by an extracellular matrix containing cellulose (15, 19, 20). A gene
cluster likely involved in cellulose synthesis was also recently identified (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
80 conditions but only when 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 an
aerobic minimal medium, suggesting a protective role against some aspect of aerobic
metabolism. Furthermore, we found that NADH dehydrogenase 2 (NADH DH), a key enzyme in
85 aerobic respiration, is important for survival in an aerobic minimal medium, providing a
physiological role for this enzyme which has otherwise been found to be detrimental for growth
in aerobic rich media (8-10).

Results

***Z. mobilis* ZM4 forms flocs in an aerobic minimal 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, hereon referred to as ZM4, under aerobic conditions in *Zymomonas* minimal medium with NH₄Cl as the sole nitrogen source (ZYMM), we observed that ZM4 formed flocs, 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 in PYG (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 bottom of test tubes but are easily observed after swirling the tube (Fig. 1A). Flocs then settle again within seconds. To quantify the extent of flocculation, we reasoned that 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; 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 dispersion of flocs when combined with cellulase (Fig. 2A). Adding cellulase and proteinase K together decreased the rate of turbidity increase, likely due to proteinase degrading 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 seconds, 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 moving forward 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 ZMO1083-1086 (*bcsABCZ*) was annotated to encode a cellulose synthase complex (Fig. 3A) (24). ZMO1086, encoding BcsZ, had previously been purified and its endoglucanase activity 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 the ability of three ZM4 mutants, each containing a transposon (Tn) insertion in a different gene in this cluster, for their ability to form flocs in aerobic ZYMM. None of the mutants formed flocs in this aerobic minimal medium (Fig. 3B). We chose to complement the BcsZ::Tn mutant since it is the terminal gene in the operon and thus is the least likely to have polar effects from the Tn insertion. Expressing *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-diguanylate monophosphate (c-diGMP) (26). c-diGMP is produced by DGCs, typically having a GG(D/E)EF domain and is degraded by phosphodiesterases (PDE), often having an EAL domain (27). The ZM4 genome has five genes annotated to have GG(D/E)EF and/or EAL domains (Fig. 4A). We examined a Tn mutant for each of the five genes for flocculation in ZYMM. Only a Tn insertion in gene ZMO0919, encoding a predicted DGC hereon referred to as DGC₀₉₁₉, prevented floc formation (Fig. 4B). Floc formation was restored in the DGC₀₉₁₉::Tn mutant by expressing ZMO0919 under its native promoter from a plasmid, whereas an empty vector did not restore flocculation (Fig. 4B). Based on these observations, we conclude that DGC₀₉₁₉ is required for floc formation, likely through the production of c-diGMP.

Mutants incapable of floc formation have low viability in an aerobic minimal medium.

Mutants that did not form flocs in the aerobic minimal medium also had final optical densities that were 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 BcsZ::Tn mutant viable cell counts were below the detection limit (~1000 CFU/ml) after being 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 CFUs immediately after vortexing flocs in cultures that had grown from colonies in aerobic ZYMM. CFUs only developed for ZM4 and the BcsZ::Tn complemented strain but not for the BcsZ::Tn mutant with and without empty vector (data not shown; detection limit of 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 grown from colonies in anaerobic ZYMM or in aerobic PYG (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 an aerobic minimal medium.

We questioned whether floc formation was stimulated by the aerobic environment or 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 an aerobic rich medium, *Z. mobilis* NADH DH mutants have been reported to behave as if they are under anaerobic conditions, exhibiting improved growth and a high ethanol yield (8-10)

instead of 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 a higher ethanol yield in aerobic PYG (Fig. 6). These trends could be attributed to the loss of *ndh* since expressing *ndh* from a plasmid in a Ndh::Tn mutant resulted in a lower final OD and acetic acid and acetaldehyde accumulation in aerobic PYG similar to ZM4 (Fig. 6).

Contrary to what we observed in PYG medium, the Ndh::Tn mutants formed visible flocs in aerobic ZYMM, though to a lesser extent than in 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. However, unlike in aerobic PYG, Ndh::Tn culture growth was less than that of ZM4 in aerobic ZYMM. This trend of poor growth was exaggerated upon transfer of dispersed Ndh::Tn mutant cultures to fresh medium (Fig. 7B, C). Expression of *ndh* from a vector improved survival of the 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 an aerobic rich medium like PYG, it is important for survival in an aerobic minimal medium.

The poor viability of NADH DH mutants in aerobic ZYMM rules out several possibilities that could have explained the low viability of the flocculation mutants, DGC₀₉₁₉::Tn and BcsZ::Tn. First, since NADH DH mutants produce little acetic acid, flocculation mutant death was unlikely due to acidification from acetic acid accumulation. In further support of this notion, supplementing aerobic ZYMM with 100 mM MOPS, pH7, did not stimulate growth of DGC₀₉₁₉::Tn or BcsZ::Tn mutants, despite partially alleviating the pH drop (Fig. 8). Second, because NADH DH mutants produce little acetaldehyde, flocculation mutant death was unlikely 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 in 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 a 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 on 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 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 the Ndh::Tn mutant (Fig10A, B).

220 We then screened several possibilities for why supplements like 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 concentration of arginine, aspartate, glutamate, methionine, and proline in yeast extract (38). The mixed amino acid supplement did not improve growth trends, nor did increasing the mineral
225 concentration 10-fold over what is normally provided in ZYMM (Fig. 10A). Cysteine is a potent antioxidant and is commonly used to scavenge O₂ in anaerobic media (37). Although 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 compared to adding the amino acid
230 mixture or a 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 of what is typical of yeast extract (39). The vitamin supplement stimulated aerobic growth of all strains, notably with the Ndh::Tn mutant showing a higher final OD, similar to what was observed in aerobic PYG (Fig.
235 10a vs. Fig. 6B).

To determine if vitamins improved survival of oxygen-sensitive mutants, we then transferred a 1% inoculum of stationary phase-cultures to fresh identical medium with and without the supplements. While all strains grew to a higher final OD with vitamins than without, the two
240 flocculation mutants did not grow as much as when inoculated from colonies (Fig. 10C vs Fig. 10A). Thus, while the vitamin supplement promoted 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 what conditions might protect *Z. mobilis* during
245 aerobic growth by examining a chemogenomic profiling database in which the fitness effect from Tn disruptions of nearly every *Z. mobilis* ZM4 gene was 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
250 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.
255 For example, the chemogenomic profiling was carried out in 10-ml volumes (41), which might have limited aeration compared to 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-
260 complementation, rescuing mutants that would otherwise be subject to the toxic aspects of aerobic growth if grown as a clonal population.

Discussion. We have demonstrated that the ability to form flocs and the aerobic respiration enzyme NADH DH are important for *Z. mobilis* ZM4 survival in an aerobic defined medium. Floc formation and respiration could work in concert; by concentrating respiratory activities, the community of cells in a floc might create a protective anaerobic microenvironment. The importance of cells clustering might also help explain why flocculation mutants grew from colonies but not 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 suggests that flocs might have protective properties independent of respiration. Such protection could be similar to that afforded by exopolysaccharide secreted by *Leuconostoc mesenteroides*, which was shown to protect cells from reactive oxygen by lowering the dissolved O₂ concentration, although the biochemical mechanism by which this occurs remains unknown (42). One possible contributing factor to the poor growth of the NADH DH mutant upon transfer could stem from having transferred cultures after turbidity had leveled off, indicating a cessation of growth. There is evidence that NADH DH activity is important for survival in starved cultures, periodically fed glucose (28). Thus, a disadvantage under starvation conditions could contribute to an explanation for why NADH DH mutants grew poorly upon transfer.

Undefined supplements promoted growth of all strains tested and in some cases inhibited floc formation (Fig. 1 and 10). We determined that mineral availability, anti-oxidant amino acids, and vitamins by themselves cannot fully explain why supplements like yeast extract have a protective role (Fig. 10). One possibility is that yeast extract contributes to a reduced environment as yeast extract has been shown to lower the oxidation-reduction potential of media (39); components in yeast extract could help to keep critical cell components in a reduced state during aerobic growth. Redox-active B vitamins in yeast extract, could contribute to this role (39, 43), and could also help explain why adding vitamins alone promoted aerobic growth in ZYMM (Fig. 10). Another possibility is that yeast extract and vitamins provides a critical nutrient that *Z. mobilis* has difficulty synthesizing in an aerobic environment, perhaps due to an O₂-sensitive enzyme.

Use of yeast extract in studies of *Z. mobilis* aerobic respiration have also led to observations by others that NADH DH activity, and respiration in general, is dispensable and even comes with a fitness cost during growth (8-10, 30). These observations raise questions as to how genes that seemingly come with a fitness cost, like that encoding NADH DH, have been maintained in *Z. mobilis*. Our observations showing that NADH DH is important for survival in an aerobic defined medium (Fig. 7) suggest that aerobic respiration is important for survival in natural environments that are deficient in protective factors like those found in yeast extract.

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

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, UC 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 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 aerobic or 10 ml anaerobic liquid media and incubated at 30°C with shaking at 225 rpm. ZYMM with 50mM glucose, 100 nM calcium pantothenate and 10 mM NH₄Cl was used as the minimal medium (4). Where indicated, cultures were also supplemented with a mixture of amino acids with final concentrations of 0.1 mg/ml for aspartate, arginine, methionine, and proline, and 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 100X stock solution described elsewhere (40). Unless stated otherwise, aerobic test tubes had loose-fitting caps to allow for air exchange. For anaerobic conditions, media was bubbled with N₂ gas and then tubes were 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 algorithm BPROM (www.softberry.com) (45) and to introduce flanking ScaI and KpnI restriction sites. PCR products were then digested and ligated into pSRKTc that had been cut with the same enzymes. The ligation reaction was transformed into *E. coli* NEB10β (NEB) and plated on 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 (46).

Cellulase and mechanical dispersion of flocs. All vortexing levels refer to that for a VWR Mini Vortexer. Pre-dispersion turbidity was assessed after vortexing cultures at level 3 for 5 seconds. To enzymatically disperse flocs, 50 µL of *Aspergillus niger* cellulase (MP; 1 % w/v) in 0.1 M MES Buffer, pH 3.5 was added per 1 mL of culture and incubated overnight at 30°C. Treated cultures were then vortexed at for 5 seconds at speed 6 prior to measuring OD₆₆₀ measurement. To mechanically disperse flocs, cultures were vortexed with 10 pulses at level 10. Dispersion was performed after the turbid fraction had reached a maximum OD₆₆₀ value, typically within six days, although sometimes longer for slow-growing strains harboring plasmids. Culture turbidity was assayed by optical density at 660 nm (OD₆₆₀) using a Genesys 20 visible spectrophotometer (Thermo-Fisher, Pittsburgh, PA).

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

Scanning electron microscopy. Flocculent culture was added to a 0.1% poly-L-lysine treated glass coverslip and incubated at room temperature for 5 min. Cells were then fixed with 3% glutaraldehyde in phosphate buffered saline (PBS) and incubated at 4°C for 1 h. Fixative was
360 removed with three washes of 4°C PBS. 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 then critical point dried using CO₂ in a Balzers CPD 030 critical point drier. The dried sample was then placed
365 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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Table 1. Strains, plasmids and primers. Kn^R, kanamycin resistance cassette; Tc^R, tetracycline resistance cassette; DGC, diguanylate cyclase; PDE, phosphodiesterase; Tn5, transposon Tn5. ZMO#### refers to a *Z. mobilis* gene number (21).

Strain; designation in text	Genotype; gene product	Source
ATCC 31821; ZM4	Wild type	(44)
UP8_C10; BcsA::Tn	ZMO1083::Tn5, Kn ^R ; cellulose synthase catalytic subunit BcsA	(44)
DN23_B1; BcsC::Tn;	ZMO1085::Tn5, Kn ^R ; cellulose synthase C-domain protein BcsC	(44)
UP4_H7; BcsZ::Tn	ZMO1086::Tn5, Kn ^R ; endoglucanase BcsZ	(44)
DN8_H11; DGC/PDE ₀₄₀₁ ::Tn	ZMO0401::Tn5, Kn ^R ; DGC/PDE	(44)
DN12_H6; DGC ₀₉₁₉ ::Tn	ZMO0919::Tn5, Kn ^R ; DGC	(44)
DN36_C3; DGC/PDE ₁₀₅₅ ::Tn	ZMO1055::Tn5, Kn ^R ; DGC/PDE	(44)
DN18_A11; DGC ₁₃₆₅ ::Tn	ZMO1365::Tn5, Kn ^R ; DGC	(44)
DN9_H1; PDE ₁₄₈₇ ::Tn	ZMO1487::Tn5, Kn ^R ; PDE	(44)
DN25_A11; Ndh::Tn	ZMO1113::Tn5, Kn ^R ; NADH dehydrogenase 2	(44)

UP8_F2; Ndh::Tn	ZMO1113::Tn5, Kn ^R ; NADH dehydrogenase 2	(44)
UP10_D3; Ndh::Tn	ZMO1113::Tn5, Kn ^R ; NADH dehydrogenase 2	(44)
Plasmid	Description	
pSRKTc	Empty vector (EV), 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	Sequence (5' → 3'); Restriction site	
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	aaaggtacccttgccaatcctcataaaaaaatgatag	This study.
pSRKTc-Ndh_For_KpnI	aaaggtaccgacgggaaaagaagccaaaggtca	This study.
pSRKTc-Ndh_Rev_SacI	aaagagctccctttggtctttcttttaaaaaggctcattact	This study.

Figures and Legends

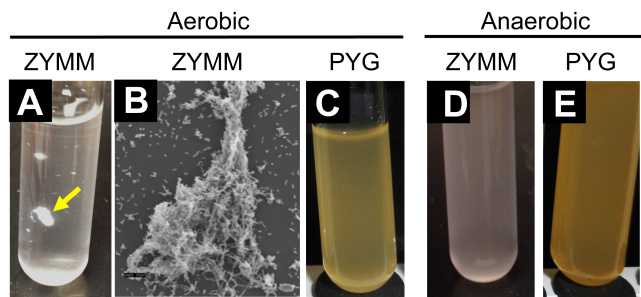


Fig. 1. Flocs are only observed in an aerobic minimal medium. (A, C-E) Photos of *Z. mobilis* ZM4 cultures in test tubes with either a minimal medium, (ZYMM), or in undefined rich PYG medium. Media were swirled by hand prior to taking a photo. (A) The yellow arrow points to a macroscopic floc. (B) Scanning electron micrograph of a floc grown in aerobic ZYMM with NH₄Cl. Magnification is 1000X at 15 kV.

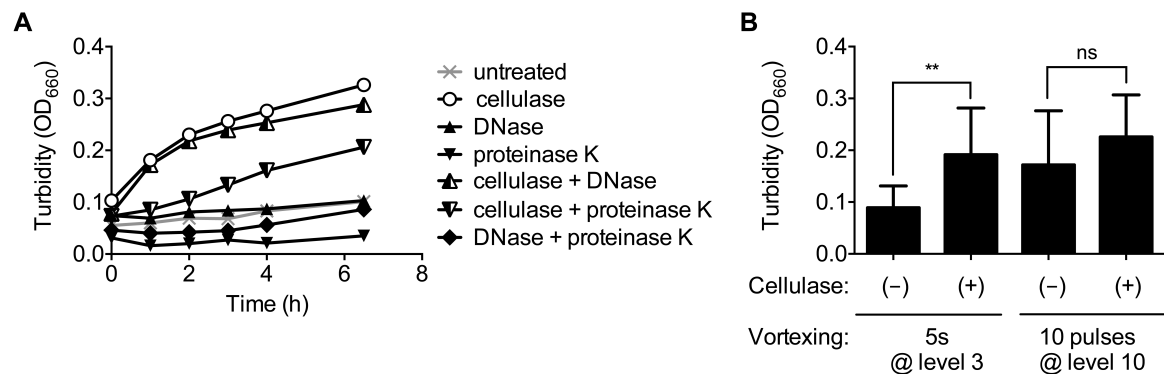


Fig. 2. Flocs can be dispersed by cellulase or harsh vortexing. (A) Treatment of ZM4 flocs with cellulase, proteinase K, and DNase. Representative trends from one of two replicates is shown. (B) Effect of gentle (5 seconds at level 3) or harsh vortexing (10 pulses at level 10) on floc dispersal with and without a 3 h cellulase treatment. Error bars, SD (n = 4). ** Significant difference (P-value < 0.01) from a one-way ANOVA with Holm-Sidak's posttest; ns, no significant difference.

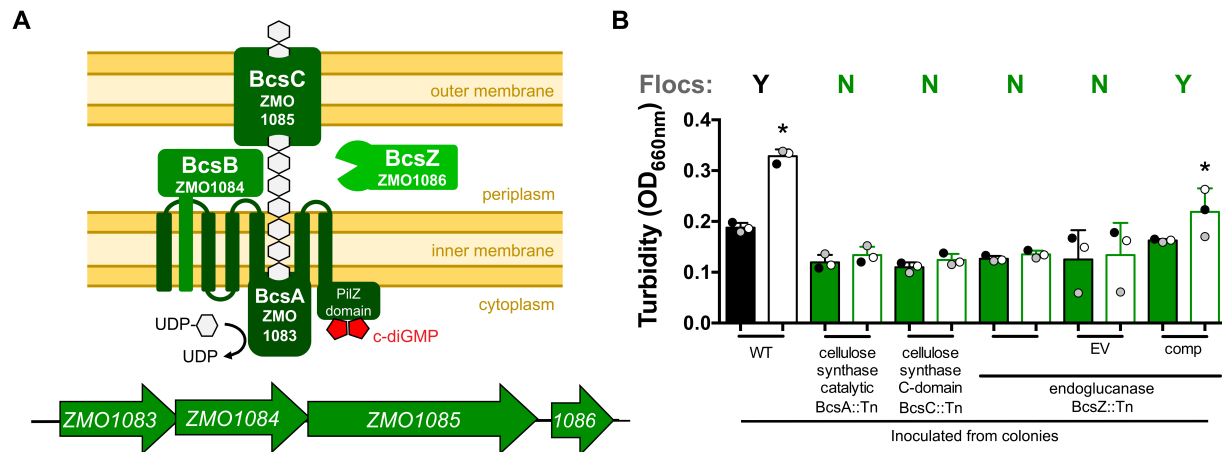


Fig. 3. Floc formation requires cellulose synthase. (A) Cellulose synthase gene cluster (bottom; ZMO1083-1086) and a schematic of the resulting protein complex (top) based on (20, 24). (B) Turbidity of aerobic cultures grown from colonies in a minimal medium (ZYMM) before (filled bars) and after (open bars) cellulase treatment. Shaded circles indicate the same culture pre- and post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N). WT, wild-type ZM4; EV, empty vector pSRKT; comp, complementation vector pSRKTc-BcsZ. Error bars, SD. *Significant difference from the corresponding filled bar (P-value < 0.1) from a one-way ANOVA with Sidak's multiple comparison posttest.



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) Turbidity 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 post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N). WT, wild-type ZM4; EV, empty vector pSRKT; comp, complementation vector pSRKTc-DGC0919. Error bars, SD. *Significant difference from the corresponding filled bar (P-value < 0.1) from a one-way ANOVA with Sidak's multiple comparison posttest.

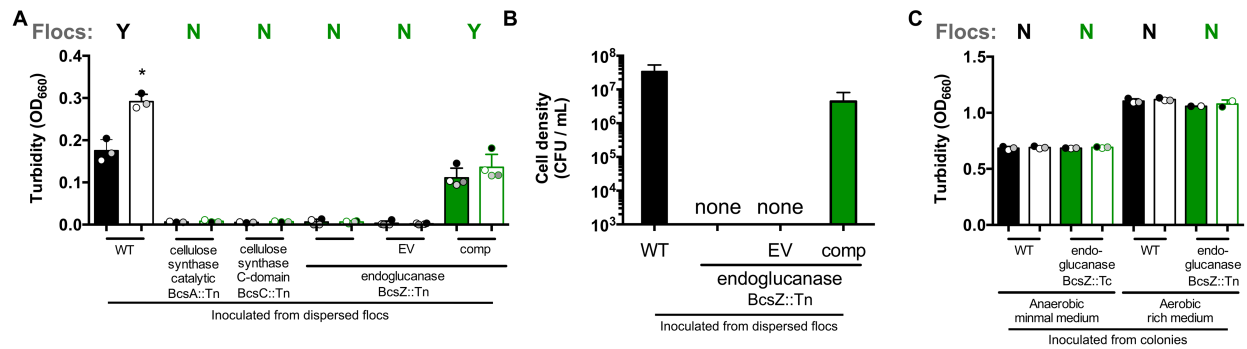


Fig. 5. Cellulose synthase genes are required for survival in an aerobic minimal medium. (A)

Cultures in aerobic minimal medium, ZYMM, inoculated from harshly vortexed cultures from conditions used in Fig. 3. (B) Colony forming units from cultures in panel A plated on PYG agar. 'None' indicates that no colonies were observed in 10 μ l of a 10⁻¹ dilution. (C) Cultures in anaerobic ZYMM or aerobic PYG medium inoculated from colonies. (A, C) Turbidity of cultures after gentle vortexing (filled bars; 5 s, level 3) and after dispersion of flocs by harsh vortexing (open bars; 10 pulses, level 10). Shaded circles indicate the same culture pre- and post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N). *Significant difference from the corresponding filled bar (P-value < 0.1) from a one-way ANOVA with Sidak's multiple comparison posttest. WT, wild-type ZM4; EV, empty vector pSRKTc; comp, complementation vector pSRKTc-BcsZ. (A-C) Error bars, SD.

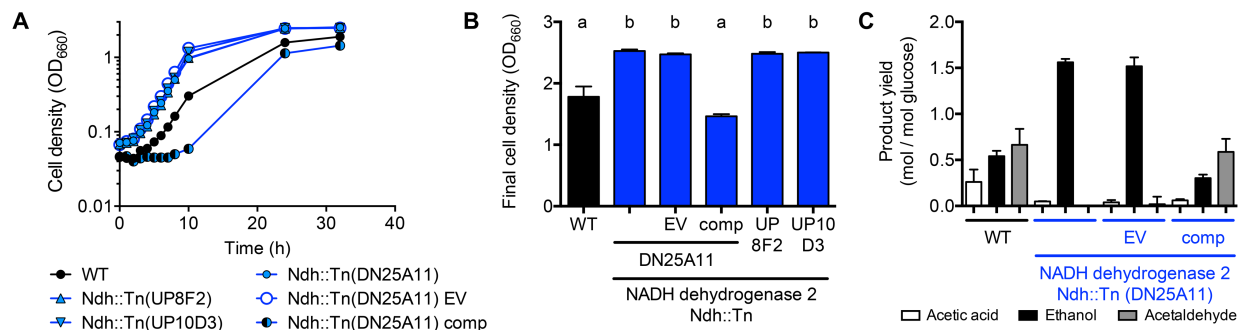


Fig. 6. NADH DH mutants show improved growth trends and a higher ethanol yield in rich PYG

medium. Representative growth curves (A), final OD values (B) and major fermentation product yields for wild-type ZM4 (WT) and NADH DH mutants (Ndh::Tn) in PYG medium. EV, empty vector pSRKTc; comp, complementation vector pSRKTc-Ndh. Error bars, SD; n=2. (A) Similar trends were observed for at least one other biological replicate.

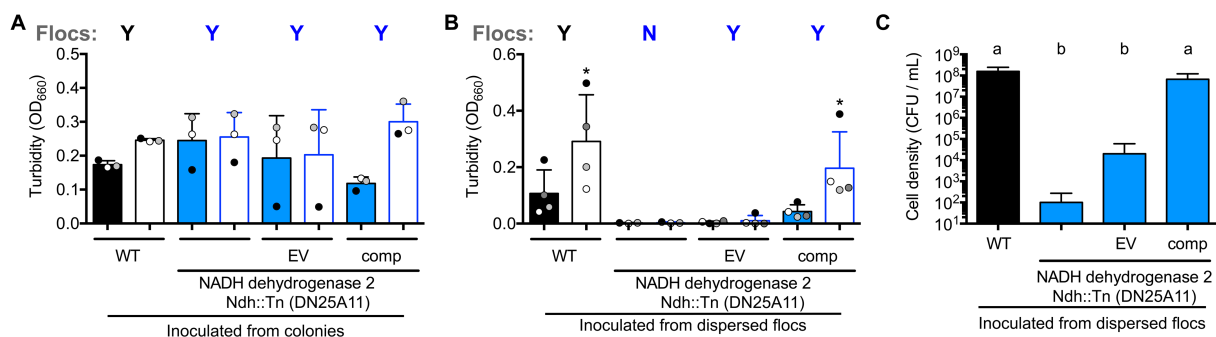


Fig 7. NADH DH is important for survival in an aerobic minimal medium. (A, B) Turbidity of cultures grown in an aerobic minimal medium (ZYMM) after gentle vortexing (filled bars; 5 s, level 3) and after dispersion of flocs by harsh vortexing (open bars; 10 pulses, level 10). Cultures were inoculated from colonies (A) or inoculated from harshly vortexed cultures from conditions in panel A (B). Shaded circles indicate the same culture pre- and post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N). *Significant difference from the corresponding filled bar (P-value < 0.1) from a one-way ANOVA with Sidak's multiple comparison posttest. (C) Colony forming units from cultures in panel B plated on PYG agar. Bars with different letters are significantly different (P-value < 0.) as determined by a one-way ANOVA with Sidak's multiple comparison posttest. (A-C) Error bars, SD. EV: empty vector pSRKTc; comp: complementation vector pSRKTc-Ndh.

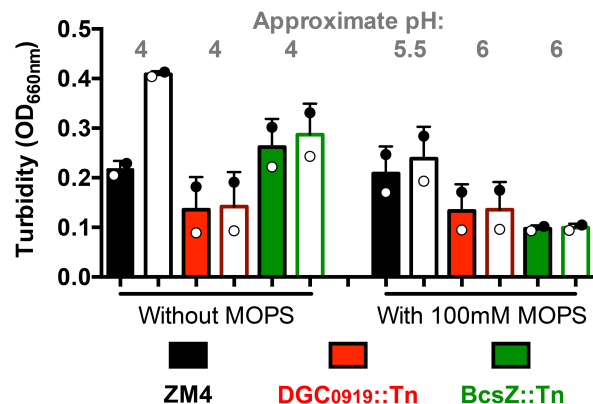


Fig. 8. Adding MOPS buffer does not promote growth in an aerobic minimal medium. Turbidity of cultures grown from colonies in an aerobic minimal medium (ZYMM) without or with 100 mM MOPS after gentle vortexing (filled bars; 5 s, level 3) and after harsh vortexing (open bars; 10 pulses, level 10) to disperse any flocs. Shaded circles indicate the same culture pre- and post-dispersion.

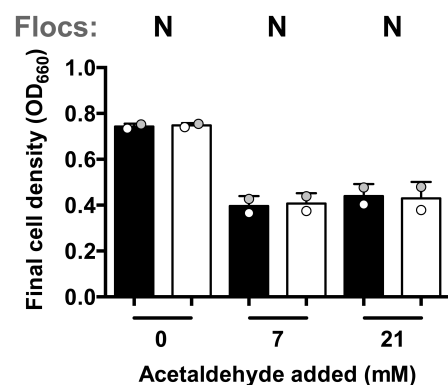


Fig. 9. Acetaldehyde does not induce floc formation under anaerobic conditions. Turbidity of ZM4 cultures grown in an anaerobic minimal medium (ZYMM) with the indicated amount of acetaldehyde after gentle vortexing (filled bars; 5 s, level 3) and after harsh vortexing (open bars; 10 pulses, level 10) to disperse any flocs. Shaded circles indicate the same culture pre- and post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N).

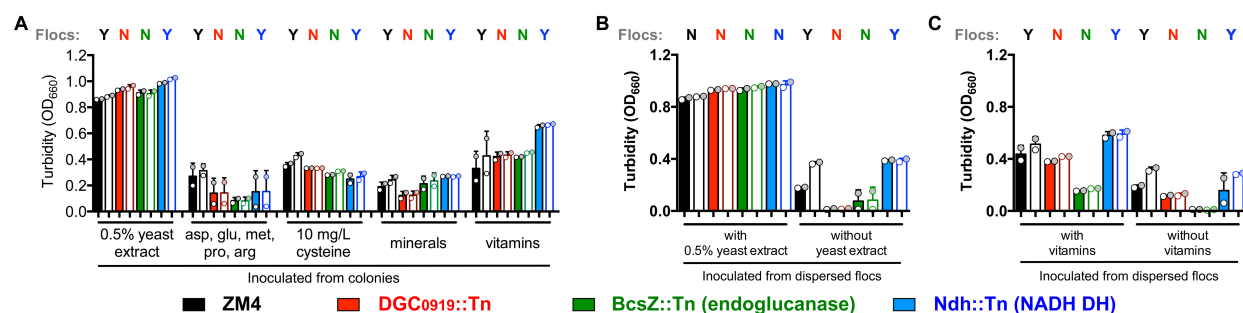


Fig. 10. Yeast extract and vitamins promote growth of oxygen-sensitive mutants. Turbidity of cultures grown in an aerobic minimal medium (ZYMM) with the indicated supplement after gentle vortexing (filled bars; 5 s, level 3) and after dispersion of flocs by harsh vortexing (open bars; 10 pulses, level 10). Cultures were inoculated from colonies (A) or inoculated from harshly vortexed cultures from panel A (B, C). Shaded circles indicate the same culture pre- and post-dispersion. Y/N indicates visual observation of flocs (Y) or not (N).

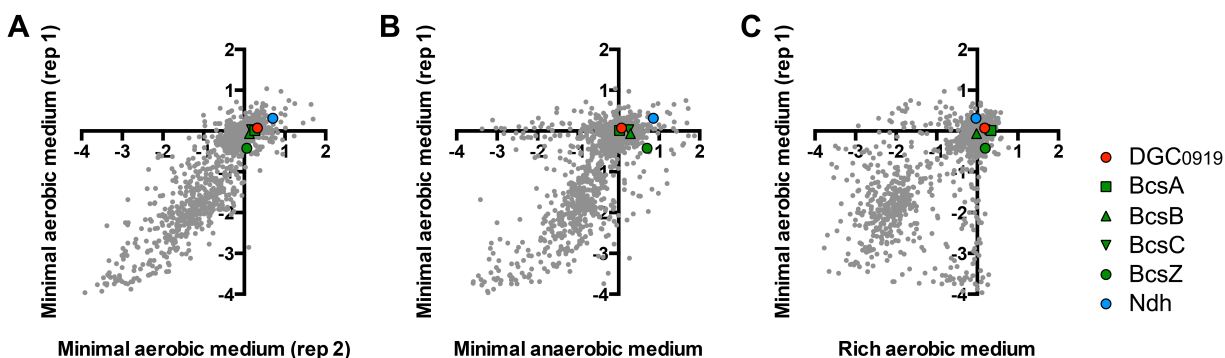


Fig. 11. Comparison of gene fitness values from a chemogenomic profiling study (41). X and Y-axis values are gene fitness values for the indicated growth condition. Gene fitness values reflect the change in population frequency for a given Tn mutant from the time of inoculation until the end of the experiment (6-8 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 grey dot is the gene fitness value for a given ZM4 gene. Each graph compares gene fitness values in an aerobic minimal medium (rep 1) with a replicate in an identical growth condition (A; control; rep 2), an anaerobic minimal medium (B) or an aerobic rich medium (C).