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Antimicrobial Activity of *Zymomonas mobilis* Is Related to Its Aerobic Catabolism and Acid Resistance

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Abstract: *Zymomonas mobilis* is an ethanologenic, facultatively anaerobic alpha-proteobacterium, known for its inhibitory effect on the growth of a wide variety of microorganisms. This property might be interesting for the design of novel antimicrobials, yet it has negative implications for biotechnology, as it hinders the use of *Z. mobilis* as a producer microorganism in cocultivation. So far, the chemical nature of its inhibitory compound(s) has not been established. In the present study, we demonstrate that the putative inhibitor is a low-molecular-weight (below 3 kDa), thermostable compound, resistant to protease treatment, which is synthesized under aerobic conditions in *Z. mobilis* strains via the active respiratory chain. It is also synthesized by aerated nongrowing, glucose-consuming cells in the presence of chloramphenicol, thus ruling out its bacteriocin-like peptide nature. The inhibitory activity is pH-dependent and strongly correlated with the accumulation of propionate and acetate in the culture medium. Although, in *Z. mobilis*, the synthesis pathways of these acids still need to be identified, the acid production depends on respiration, and is much less pronounced in the non-respiring mutant strain, which shows low inhibitory activity. We conclude that propionate and acetate play a central role in the antimicrobial effects of *Z. mobilis*, which itself is known to bear high resistance to organic acids.

Keywords: *Zymomonas mobilis*; antimicrobial activity; aerobic catabolism; acid resistance

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1. Introduction

Zymomonas mobilis is a facultatively anaerobic alpha-proteobacterium, with an efficient and rapidly operating homoethanol fermentation pathway. Its outstanding ethanol productivity, exceeding that of yeast *Saccharomyces cerevisiae* by more than a factor of three [1], together with remarkable tolerance to high ethanol and sugar concentrations, has attracted the interest of the biotechnological research community over several decades.

The Entner–Doudoroff pathway, in combination with pyruvate decarboxylase and alcohol dehydrogenases, form the backbone of the relatively simple *Z. mobilis* central metabolic network [2]. The Krebs cycle and the pentose phosphate pathway are incomplete, and the glyoxylate shunt is absent [3,4]. In addition, this bacterium possesses an active aerobic respiratory chain with oxygen consumption rates comparable to those of *Escherichia coli* or *Corynebacterium glutamicum*, albeit with atypically low degree of energy coupling [5–7]. In order to fully exploit its biotechnological potential, metabolic engineering aims to broaden the substrate and product spectra of *Z. mobilis* beyond the mere bioethanol synthesis with glucose, fructose, or sucrose-containing substrates [8–10]. Eventually, this implies the design of novel bioprocesses, involving cocultivation with other producer microorganisms. However, *Z. mobilis* demonstrates antimicrobial activity via an unknown mechanism, which might be useful for the treatment of infections, but may hamper cocultivation.

Antagonistic interactions between *Z. mobilis* and a wide variety of other microorganisms have previously been reported in a number of publications (for a review, see [11]). Early reports stated that it inhibited the growth and conjugative ability of *E. coli* mating partners [12]. It was observed that this bacterium might be producing some unidentified antimicrobial substances ('zymocins') in studies of the therapeutic potential of *Z. mobilis*, and, from its application in cocultivation bioprocesses. *Z. mobilis* has proved to be efficient in treating infections [13–15]. The spectrum of its activity is broad, acting against bacteria, fungi, and protists. In animal models, *Z. mobilis* culture was shown to have probiotic properties and to protect against sepsis by alleviating the burden of pathogens and via immunomodulatory effects [16]. Evidence has been accumulated on the ability of *Z. mobilis* to compete with other microorganisms in bioprocesses. For example, a fermenter with glass beads initially colonized by culture of *Z. mobilis* remained essentially free of contaminants for over 4 months of nonsterile operation [17]. During cocultivation with the yeast *Pichia stipitis* on a glucose and xylose mixture, *Z. mobilis* was shown to inhibit yeast cell growth and its ability to ferment xylose [18].

Lima et al. [19] hypothesized that *Z. mobilis* might be producing an antimicrobial peptide, yet did not purify and characterize it. Thus, the chemical nature of the *Zymomonas* antimicrobial activity remains unresolved. In the present study, we aimed to identify the antimicrobial compound(s), as well as the strains and culture conditions, with the maximum antimicrobial effect. We could not find evidence in support of the peptide nature of the antimicrobial activity, yet we identified acetate and propionate as the principal *Z. mobilis* antimicrobial compounds, and we showed the respiratory metabolism of the aerobic culture to facilitate both accumulation of these acids and antimicrobial activity. We suggest that the relatively high resistance of *Z. mobilis* to organic acids, representing an advantage in strains engineered for growth on lignocellulosic hydrolysates [20,21], is the physiological prerequisite of such antimicrobial activity.

2. Materials and Methods

2.1. Strains, Media and Cultivation

Two wildtype *Z. mobilis* strains, Zm6 (ATCC 29191) and Zm4 (ATCC 31821), as well as mutant derivatives of the strain Zm6 (the respiratory mutant strain Zm6-*ndh*, with a cm^r marker inserted in the type II NADH dehydrogenase gene *ndh* [22], the catalase-deficient strain Zm6-*cat*, with a cm^r marker inserted in the catalase gene [23], and the iron-containing alcohol dehydrogenase isozyme ADH II-deficient strain Zm6-*adhB*, with a kan^r marker inserted in the *adhB* gene [24]) were used for screening of antimicrobial activity. For testing of the antimicrobial activity of *Z. mobilis*, *Escherichia coli* strain DH5α and the chloramphenicol-resistant strain *E. coli* JM109 pGEMpdc:cm, carrying a plasmid construct with a cm^r marker, were employed. Furthermore, several representatives of the ESKAPE pathogens (*Staphylococcus aureus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, and *Enterococcus faecium*) were subjected to screen for sensitivity to *Z. mobilis* antimicrobial activity.

All *Z. mobilis* strains were routinely grown on a standard culture medium containing (per liter) 5 g of yeast extract, 1 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.5 g of $MgSO_4 \cdot 7H_2O$, supplemented with 40 mL of 50% glucose solution. *E. coli* DH5α was grown on LB medium, containing (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. The medium for the chloramphenicol-resistant strain *E. coli* JM109 pGEMpdc:cm was supplemented with chloramphenicol (25 $\mu g \cdot mL^{-1}$), while that for the chloramphenicol-resistant Zm6-*ndh* was supplemented with 100 $\mu g \cdot mL^{-1}$ chloramphenicol.

In particular experiments, other media were used for cultivation of *Z. mobilis* and the ESKAPE pathogens: (i) Mueller–Hinton broth (Condalab), containing per liter 17.5 g of acid casein peptone, 2 g of beef infusion, and 1.5 g of corn starch; (ii) R2A Agar (Biolife), containing per liter 0.5 g of yeast extract, 0.5 g of proteose peptone, 0.5 g of acid digest of casein, 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.024 g of $MgSO_4$, 0.3 g of sodium pyruvate, and 14 g of agar; (iii) RM broth, containing per liter 10 g of yeast extract and 2 g of KH_2PO_4.

Z. mobilis cultivation was carried out in 500 mL shaken flasks with a 100 mL working volume, at 30 °C and 180 rpm. Additional sparging with atmospheric air with a flow rate up to 0.5 L·min^{-1} (5% *v*/*v*/*m*) was used during cultivation. Laboratory-scale *Z. mobilis* fermentations were carried out in 2 L BIOSTAT A™ bioreactors (Sartorius™, Goettingen, Germany) with a working volume of 500 mL at 30 °C. Where needed, pH was controlled by automated addition of NaOH. Anaerobic conditions were set by gassing the culture with nitrogen gas.

2.2. Fractionation and Concentration of Culture Medium

The *Z. mobilis* supernatant was prepared by centrifugation of bacterial cultures for 10 min at 7000 rpm and removing the supernatant from the bacterial pellet. For use in subsequent separation experiments, the supernatant was concentrated via lyophilization. Then, 5 mL of the frozen (−80 °C) supernatant sample aliquots were lyophilized for 24–32 h at −90 °C using a ScanVac CoolSafe freeze-dryer until complete removal of ice by sublimation. The dry lyophilized material was resuspended in 0.2 M citrate buffer (citric acid), 0.1 M phosphate (KH_2PO_4) buffer, or 0.1 M TEAE (triethanolamine hydrochloride) buffer with varying pH to reach 10× concentration of the starting material.

For separation of putative small peptides in the supernatant of fermentation medium, we used size-exclusion chromatography on a Sephadex G-25 (superfine) column, since its fractionation range of 1000–5000 Da would be appropriate for this objective. We used a XK26/20 (Cytiva) column with diameter of 25 mm and height of 120 mm, packed with 60 mL of matrix. The flow rate was 5 mL/min, with the fraction size typically being 5 mL. The column was previously equilibrated with 0.2 M citrate or phosphate buffer, pH 5.

Amicon® Ultra-15 Centrifugal Filter Units with 10 kDa and 3 kDa cutoff were used for determination of the antimicrobial compound size. First, 12 mL of ZM4 supernatant was transferred to the 10 kDa cutoff filter unit and centrifuged for 1 h at 4500 rpm. The retentate was saved for antimicrobial activity testing, and 5 mL of the permeate was transferred to the 3 kDa cutoff filter unit and repeatedly centrifuged for 1 h at 4500 rpm. Both the retentate and the permeate were saved for antimicrobial activity quantification.

2.3. Quantification of the Antimicrobial Activity

2.3.1. Inhibition Zone

The *Z. mobilis* strains were screened for their antimicrobial activity using the agar overlay method, basically following the procedure of Haffie et al. [12]. On agarized standard culture medium, 3 µL of *Z. mobilis* overnight cultures were inoculated and grown overnight at 30 °C until the colonies were visible. Then, an *E. coli* suspension was prepared by suspending *E. coli* colonies in double-distilled (dd) H_2O until an OD_{600} of about 0.4. Then, 2 mL of melted R2A agar was mixed with 1 mL of the *E. coli* cell suspension, and the mixture was poured over the *Z. mobilis* plates. The plates were incubated at 37 °C. The inhibition zone radii were measured from the middle of the *Z. mobilis* colonies.

2.3.2. Optical Density Measurements and Quantification of Growth in Liquid Media

To determine the antimicrobial activity of the *Z. mobilis* culture supernatants, *E. coli* growth curves were obtained in the presence of *Z. mobilis* supernatant in a microplate (96-well plate) reader Infinite® M200 PRO Multimode Microplate Reader (Tecan, Maennedorf, Switzerland). First, 100 µL of 2× concentrated LB broth with inoculated *E. coli* DH5α cells (1:100 *v*/*v*) was mixed with the *Z. mobilis* culture supernatant or its separate fractions. Antimicrobial activity was examined at several test material concentrations, by adding 25, 50, or 100 µL of supernatant (yielding 12.5%, 25%, or 50% *v*/*v* of the supernatant final concentration, respectively). The total working volume in each well was 200 µL; therefore, when necessary, dd H_2O was added to reach this final volume. For positive *E. coli* growth controls, 100 µL of dd H_2O was added instead of the test material. *E. coli* was incubated at 32 °C for 13–18 h. The plates were shaken at 200 rpm, and optical density measurements (λ = 600 nm) were taken at 10.5 min intervals.

For quantitative comparison of the obtained growth curves, the specific growth rate (μ) was calculated for each batch cultivation. The specific growth rate was calculated for the exponential growth phase (thus corresponding to maximum specific growth rate) according to the following equation:

$$\mu = \ln\left(\frac{OD_2}{OD_1}\right) / (t_2 - t_1) \quad (1)$$

where OD_1 is the OD_{600} at the beginning of the exponential phase, and OD_2 is the OD_{600} value at the end of it; t_1 and t_2 are the respective timepoints of the optical density measurements. The calculated values were relevant only for the specific experimental conditions, where higher μ_{max} values indicated a lower antimicrobial activity of the test material. To compare the *Z. mobilis* antimicrobial activity toward different ESKAPE pathogens, the respective bacteria were cultivated in microplates as described above for *E. coli*, and their μ values were normalized to the respective specific growth rates without added *Z. mobilis* culture supernatant, which were set at 100%.

2.4. Analytical Methods

Glucose and ethanol concentrations were measured by high performance liquid chromatography (HPLC) Agilent 1100 series (Agilent, Santa Clara, CA, USA) with a refractive index detector (RID) using a Shodex SH1011, L column (particle size 6 μm) at 50 °C (0.005 M H_2SO_4, flow rate 0.6 mL·min^{-1}, sample size 5 μL). Organic acid concentrations were measured by HPLC (Agilent 1100 series) with diode-array detection (DAD) at 210 nm using a Supelco Supelcogel C610H column (particle size 9 μm) at 30 °C (0.1% H_3PO_4, flow rate 0.5 mL·min^{-1}, sample size 5 μL). Acetaldehyde concentration in the bacterial supernatant was measured using a Megazyme Acetaldehyde Assay Kit following the manufacturer's instructions. Protein concentration in the samples was determined using the modified Lowry procedure according to Markwell et al. [25].

2.5. Statistical Analysis

The data were statistically analyzed with the one-way analysis of variance (ANOVA) test using R (version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria). Tukey's multiple comparison test was used to determine the differences between the treatment means and controls. A p-value lower than 0.05 ($p < 0.05$) was considered to be significant. The results are expressed as the average values ± standard deviation determined from triplicate experiments.

3. Results

3.1. Z. mobilis Strains and Culture Conditions Producing Maximum AM Effect

Z. mobilis strains were screened for their antimicrobial activity by detecting the inhibition zones in the agar overlays with growing *E. coli* DH5α cells. Four of the tested strains (Zm4, Zm6, Zm6-*cat*, and Zm6-*adhB*) produced fairly similar inhibition zones around their colonies, ranging from about 7.5 mm for Zm6-*cat* to 8.75 mm for Zm4 (Table 1). A clear exception was the strain Zm6-*ndh*, which produced negligible inhibition zones. Inhibition zones of the parent strain Zm6 and its respiratory deficient derivative Zm6-*ndh* are presented in Figure 1.

Table 1. Inhibition zones in *E. coli* agar overlays with several *Z. mobilis* strains.

Strain	Inhibition Zone Radius, mm
Zm4	8.75 ± 0.50
Zm6	8.00 ± 0.82
Zm6-*adhB*	8.25 ± 0.50
Zm6-*cat*	7.50 ± 0.58
Zm6-*ndh*	3.25 ± 0.50

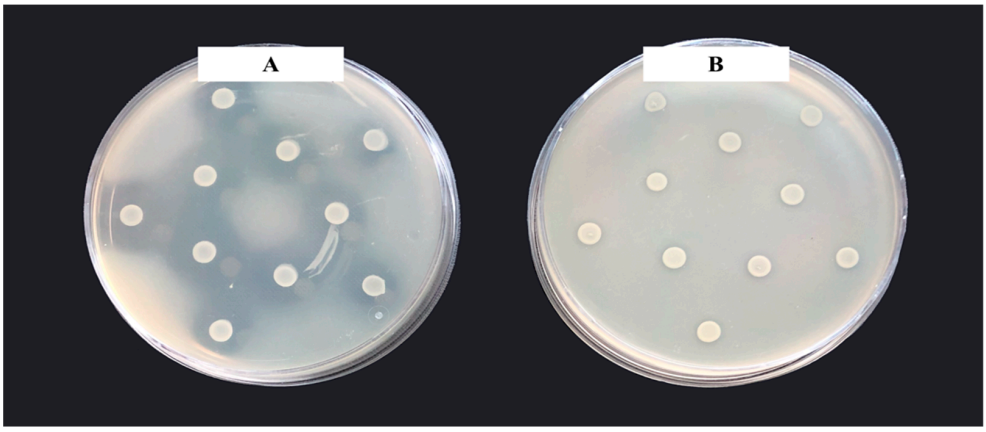


Figure 1. Inhibition zones in *E. coli* agar overlaid with wildtype *Z. mobilis* Zm6 (**A**) and its respiratory mutant derivative Zm6-*ndh* (**B**).

Supernatant from aerobically grown Zm4 culture was added to batch cultures of *E. coli* and several of the so-called ESKAPE pathogens (representatives of *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacter* species). The relative decrease in their specific growth rates was monitored (Figure 2). *Z. mobilis* cells were sedimented by centrifugation, and their culture supernatant was added to the growth medium in Tecan plates at various final concentrations (see Section 2). Strong inhibitory effects were observed with all tested bacteria. *E. coli* was chosen for further experiments as the test microbe, since it was the least pathogenic.

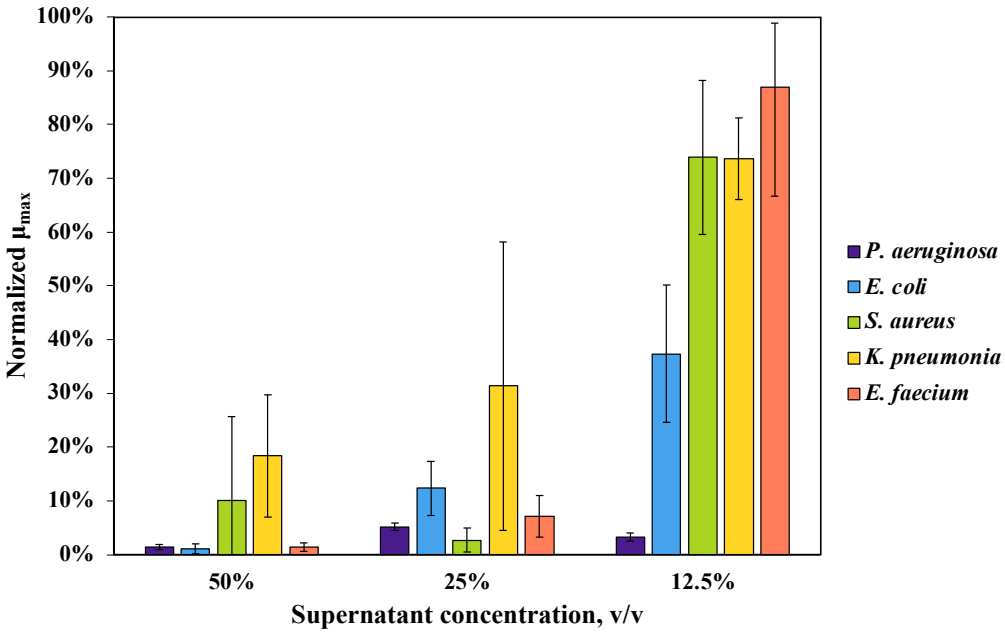


Figure 2. Effect of various concentrations of supernatant of aerobically grown Zm4 culture on the maximum specific growth rate of *E. coli* and several ESKAPE pathogens. μ_{max} values are normalized to each bacterium's positive control—the specific growth rate without Zm4 supernatant addition. The growth rate of *E. coli* and *P. aeruginosa* at 12.5% and that of all strains at higher supernatant concentrations significantly differed from their respective control values ($p < 0.001$).

In contrast to the pronounced antimicrobial effect of cultures grown under vigorous aeration, anaerobic or oxygen-limited growth of the respiring *Z. mobilis* strains Zm6 and Zm4 resulted in an almost no inhibitory effect. For the slow-respiring, respiratory NADH dehydrogenase-deficient strain Zm6-*ndh*, the inhibitory effect was also substantially smaller

under aerobic culture condition (Figure 3A). Notably, Zm6-*ndh* under aerobic conditions had a much higher ethanol yield than the wildtype, since its respiratory NADH dehydrogenase is inactivated and does not compete with the alcohol dehydrogenase reaction for NADH [22,26]. Taken together, these observations indicate that the antimicrobial effect of *Z. mobilis* is unrelated to its superb ethanol-producing capacity. For the strain Zm4, a series of cultivations at different set pO_2 concentrations were carried out in fermenters, and a strong positive correlation was observed between the inhibitory effect of supernatant on *E. coli* growth and the pO_2 level maintained during Zm4 cultivation (Figure 3B). These data show the importance of active respiratory catabolism for generation of the *Z. mobilis* antimicrobial compound(s).

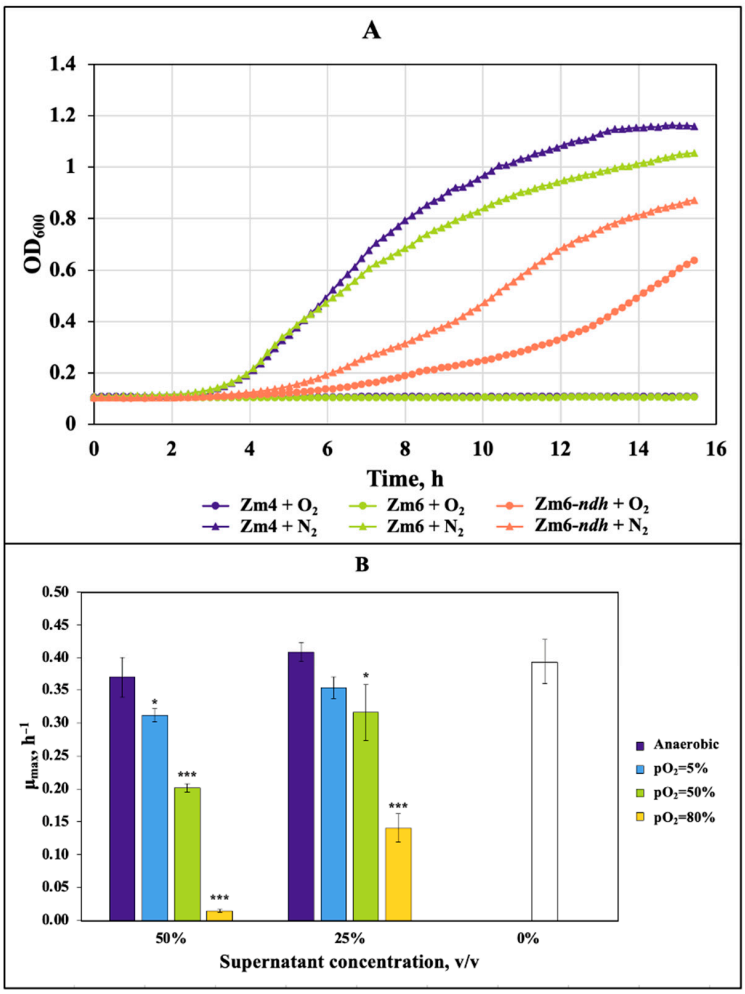


Figure 3. Effect of *Z. mobilis* culture aeration on the inhibitory effect of its culture supernatant on *E. coli* growth: (**A**) effect of *Z. mobilis* culture supernatants (at 50% final concentration) from aerobically or anaerobically cultivated strains Zm4, Zm6, and Zm6-*ndh*; (**B**) dependence of the inhibitory properties of the supernatants from Zm4 batch cultures grown in fermenters at various set pO_2 values. Empty bar, *E. coli* specific growth rate without any additions. * $p < 0.05$; *** $p < 0.001$.

Under aerobic culture conditions, respiring *Z. mobilis* produces the inhibitory compound acetaldehyde, the metabolic precursor of ethanol, due to withdrawal of NADH from the alcohol dehydrogenase reaction by the respiratory chain [26,27]. However, acetaldehyde accumulation did not explain the observed antimicrobial effect of aerobic fermentation medium. Acetaldehyde is a highly volatile compound, and vigorous gassing with air efficiently decreases its concentration in the fermentation medium. Yet, a 5 h gassing of the fermentation medium with air, which decreased acetaldehyde concentration far below its inhibitory values,

still could not eliminate most of the antimicrobial activity, as shown in Figure 4. It was, therefore, concluded that the antimicrobial compound(s) were less volatile than acetaldehyde.

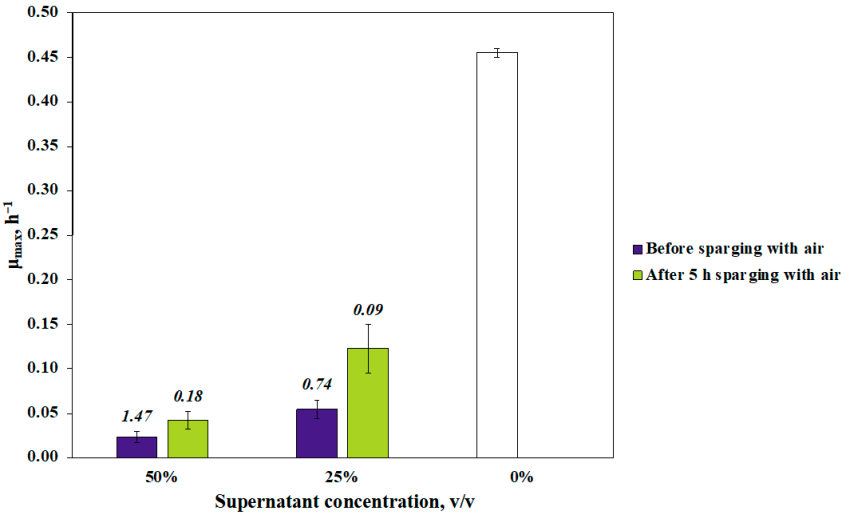


Figure 4. Effect of gassing of aerobically grown Zm4 culture supernatant with air on its growth-inhibitory properties. The acetaldehyde concentration (mM) in the *E. coli* culture media after addition of the Zm4 culture supernatant is shown on top of the bars. Empty bar, *E. coli* specific growth rate without any additions. In all cases, supernatant addition significantly decreased the growth rate ($p < 0.001$).

3.2. Properties of the Putative Antimicrobial Compound(s)

As the next step, we analyzed the effects of temperature and pH on the antimicrobial activity, the approximate molecular weight(s) of the active compound(s), and their protease sensitivity. The active compound(s) of Zm4 fermentation medium appeared to be thermally stable. Most of the inhibitory activity was retained even after 20 min of treatment of the culture supernatant at 90 °C (Figure 5).

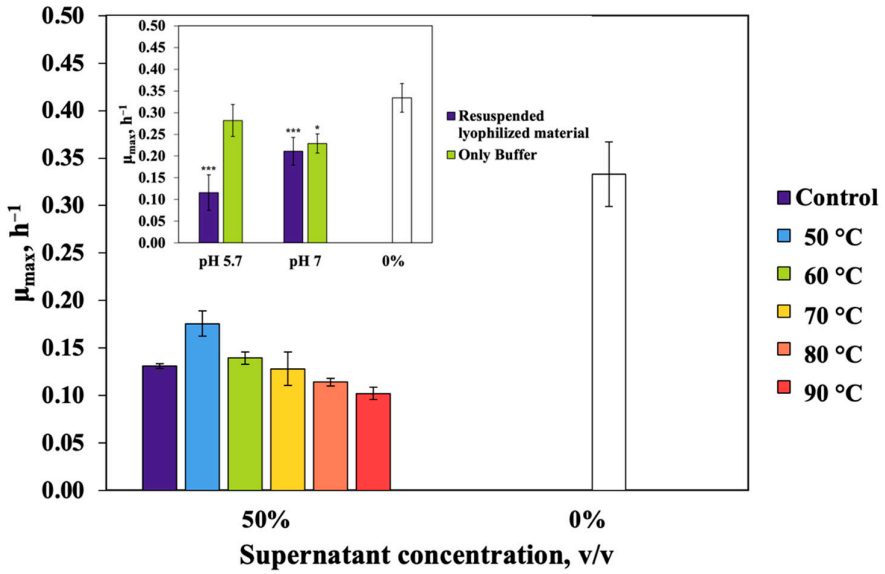


Figure 5. Effects of temperature and pH on antimicrobial activity of the Zm4 culture supernatant. Effect of thermal pretreatment at various temperatures on the *E. coli* specific growth rate. Duration of pretreatment, 20 min; control, without pretreatment. In all cases, supernatant addition significantly decreased the growth rate ($p < 0.001$). Inset: The pH dependence of the inhibitory effect of concentrated Zm4 culture supernatant in 0.2 M citrate buffer. Empty bars, *E. coli* specific growth rate without any additions. * $p < 0.05$; *** $p < 0.001$.

To examine the effect of pH on antimicrobial activity, the concentration of the antimicrobial compound in the culture supernatant was increased 10-fold by lyophilization and by subsequent resuspension of the dried powder in 0.2 M citrate buffer, with two pH values (5.7 and 7). Almost no antimicrobial activity of Zm4 culture supernatant was seen at pH 7, where there was no significant difference between the addition of 25% of citrate buffer with or without resuspended lyophilized material (Figure 5, Inset). However, at pH 5.7, which is close to the pH range (5.5–5.7) in the experiments described above (Figures 2–4), there was a strong inhibitory effect.

In order to estimate the molecular weight of the active compound(s), the fermentation medium was subjected to serial molecular cutoff filtrations, as described in Section 2. The filter with the smallest pore diameter, retaining molecules larger than 3 kDa, still resulted in antimicrobial activity in the filtrate (not shown). From that, we concluded that the molecular weight of the active compound(s) was below 3 kDa.

For testing the sensitivity to proteinases, the *Z. mobilis* supernatant was incubated with serine type proteinase K (Thermo Scientific™, Waltham, MA, USA). According to the manufacturer's specifications, the smallest peptide it can hydrolyze is a tetrapeptide. Here, 0.1 to 1 mg/mL concentrations of the proteinase in the assay were used. The samples were incubated at 50 °C for 2 h and inactivated at 70 °C for 20 min. However, the treatment with proteinase K had no measurable effect on the antimicrobial activity (not shown).

In summary, these results support the previous observations [12] that the *Z. mobilis* antimicrobial activity is related to some low-molecular-weight, protease-insensitive, and thermo-resistant compound(s). Furthermore, we found that the antimicrobial activity required a pH below 7. However, we could not repeat the results of Lima et al. [19], who reported sensitivity to the treatment with protease K and proposed that the active compound was a peptide.

3.3. Evidence That Acetic and Propionic Acids Determine the Z. mobilis Antimicrobial Activity

Insensitivity to proteases, high thermal stability, and the low molecular weight per se do not rule out the peptide nature of the antimicrobial compound(s). Such properties have been reported for low-molecular-weight bactericins (for a review, see [28]). Yet, other non-peptide inhibitory compounds might have similar properties. Because of the dependence of the antimicrobial effect on pH and the respiratory activity of *Z. mobilis* cells, we considered the byproducts of their aerobic catabolism as potential antimicrobial compounds. Since *Z. mobilis* has a truncated Krebs cycle, respiratory metabolism does not result in complete oxidation of catabolic substrate to CO_2. Instead, accumulation of acetaldehyde, acetic acid, and other minor acidic byproducts takes place, as the NADH is being withdrawn by the respiratory chain [26,27,29]. Given that (i) vigorous gassing of the medium with air efficiently removed acetaldehyde, yet had little effect on the antimicrobial activity (see above), and that (ii) antimicrobial activity depended on pH, we hypothesize short-chain fatty acids as the likely candidates for antimicrobial compound(s).

Notably, aerobic conditions stimulated the production of several acids by respiring *Z. mobilis* (Figure 6A), including acetate and propionate, for which antimicrobial activity is well established. Anaerobically cultivated Zm4 and Zm6, as well as the slow-respiring respiratory mutant strain Zm6-*ndh* with reduced antimicrobial activity, accumulated substantially less propionate and acetate. Figure 6B shows the acetate and propionate concentrations in the stationary-phase fermentation media of *Z. mobilis* cultures, which were tested for their effects on *E. coli* growth, presented in the Figure 3A. A comparison of Figures 3A and 6B reveals an association between the accumulation of acetate and propionate, and the strength of the inhibitory effect.

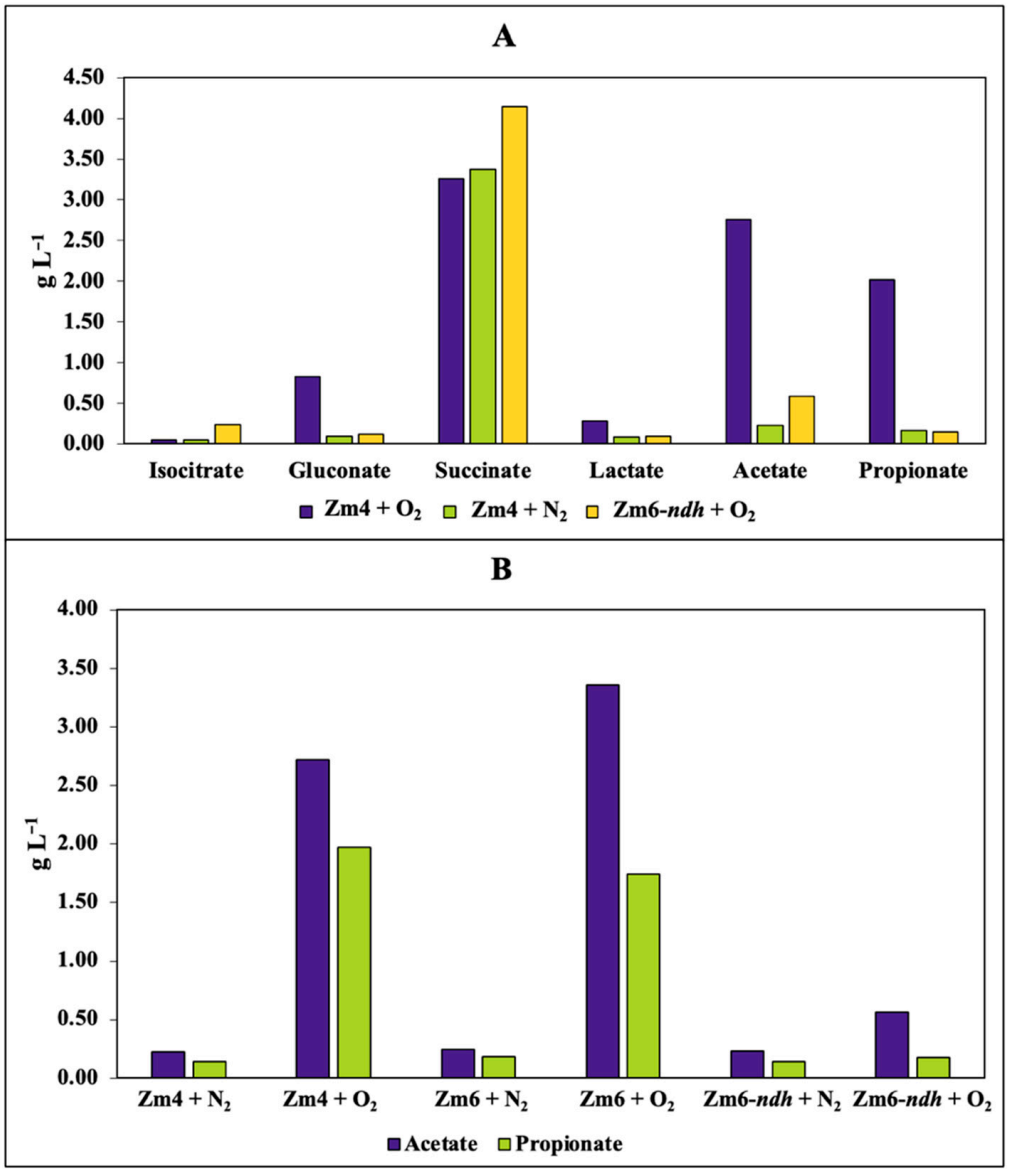


Figure 6. Acid byproducts in *Z. mobilis* stationary-phase batch cultures: (**A**) accumulation of acids in aerobically vs. anaerobically cultivated strain Zm4 and aerobically grown respiratory mutant Zm6-*ndh*; (**B**) dependence of acetate and propionate accumulation on aeration in the strains Zm4, Zm6, and Zm6-*ndh*. Representative results of one experiment in a series are presented.

During our initial attempts to verify if the antimicrobial compound was a low-molecular-mass peptide, we separated the lyophilized and 10-fold concentrated fermentation medium by size-exclusion chromatography on a Sephadex G-25 (superfine) column. After passing through the column, the antimicrobial activity of the aerobic culture of Zm4 appeared in several fractions (Figure 7A). The increased propionate and acetate concentrations in these active fractions (No. 4 to 7) mirrored their inhibitory effect on the specific growth rate of *E. coli*. In the corresponding fractions from the anaerobic culture lacking antimicrobial activity, no propionate and almost no acetate were present (Figure 7B). Thus, the size-exclusion chromatography step allowed to concentrate the active compound(s), indirectly supporting the key role of acetate and propionate in the antimicrobial effect. However, as seen in Figure 7A, the protein content was also elevated in some of the active fractions from the aerobic culture supernatant.

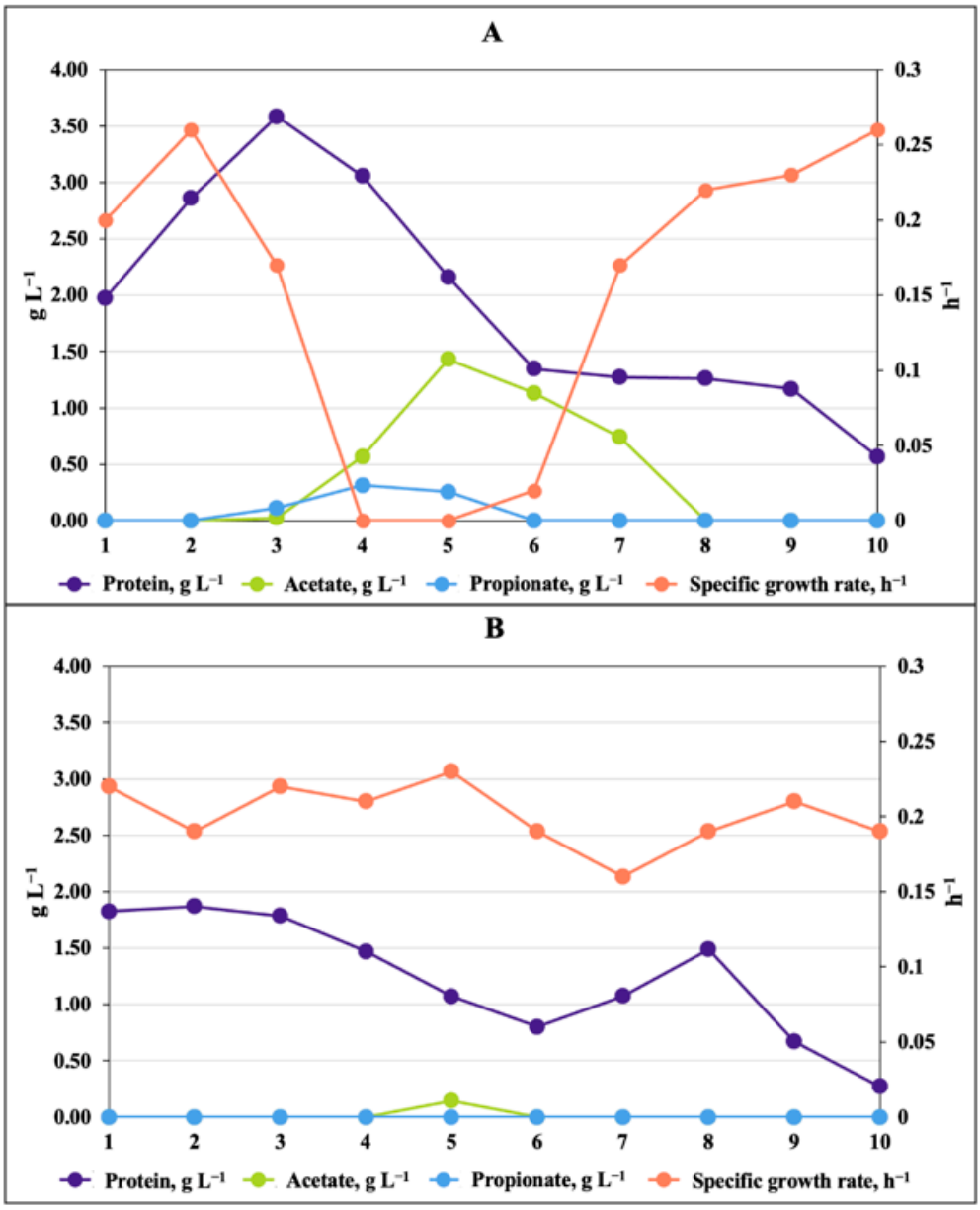


Figure 7. The composition and growth-inhibitory effect of fractions obtained by size-exclusion chromatography of lyophilized and concentrated Zm4 culture supernatant: (**A**) aerobically grown culture; (**B**) anaerobically grown culture. The specific growth rate of *E. coli* culture was assayed after addition of 100 µL of the respective fraction material per 200 µL of total working volume in a microplate well. Representative results of one experiment in a series are presented.

In order to discriminate between the peptide and nonpeptide nature of the antimicrobial effect, we examined the growth-inhibitory activity of the supernatants obtained from nongrowing, aerated, glucose-consuming *Z. mobilis* cell suspensions, incubated with or without chloramphenicol, the prokaryotic inhibitor of protein biosynthesis. The chloramphenicol-resistant *E. coli* strain was used to test for the inhibitory activity in this experiment. *Z. mobilis* Zm4 and Zm6-*ndh* cells from aerobically growing cultures were sedimented, washed, and resuspended to 8 g dry wt·L^{-1} in 100 mM phosphate buffer, pH 6, and incubated in shaken flasks with the addition of 50 g·L^{-1} glucose. The Zm4 cell suspensions gradually accumulated acetic acid (Table 2). After 2 h of incubation, the concentration of acetate in the Zm4 suspension without added chloramphenicol reached 1.8 g·L^{-1}, and that of propionate reached 0.22 g·L^{-1}. Supplementing *E. coli* culture medium

with 25% of the supernatant obtained after Zm4 cell sedimentation resulted in complete inhibition of *E. coli* growth.

Table 2. Accumulation of acetate and propionate in aerated, nongrowing *Z. mobilis* cell suspensions consuming glucose, and the effect of their incubation media on the *E. coli* specific growth rate. Chloramphenicol (cm) was added to *Z. mobilis* at 100 μg·mL^{-1} final concentration. At indicated timepoints, cells were sedimented, the supernatants were added to the *E. coli* culture medium at 25% final concentration, and growth of the chloramphenicol-resistant *E. coli* strain was monitored. Mean values of two replicates are presented.

Incubation Time (min)	Strain	Acetate (g·L^{-1})	Propionate (g·L^{-1})	*E. coli* μ (h^{-1})
1	Zm4	0.19	0.00	0.51
	Zm4 + cm	0.99	0.00	0.38
	Zm6-*ndh*	0.02	0.00	0.50
	Zm6-*ndh* + cm	0.02	0.08	0.51
10	Zm4	0.33	0.00	0.58
	Zm4 + cm	1.11	0.00	0.39
	Zm6-*ndh*	0.01	0.00	0.51
	Zm6-*ndh* + cm	0.02	0.00	0.51
30	Zm4	0.49	0.10	0.58
	Zm4 + cm	1.62	0.00	0.05
	Zm6-*ndh*	0.12	0.00	0.53
	Zm6-*ndh* + cm	0.12	0.00	0.54
120	Zm4	1.81	0.22	0.01
	Zm4 + cm	3.72	0.00	0.00
	Zm6-*ndh*	0.57	0.11	0.39
	Zm6-*ndh* + cm	0.57	0.00	0.31

With chloramphenicol, an even more pronounced antimicrobial activity was observed. Already after 30 min of incubation, the supernatant from the chloramphenicol-treated Zm4 suspension completely inhibited the growth of the chloramphenicol-resistant *E. coli* strain. For some reason, in the presence of chloramphenicol, the Zm4 suspension did not produce propionate, but accumulated more acetate than the control. Much the same as for growing cultures, for the nongrowing *Z. mobilis* cell suspensions, their inhibitory effect was associated with the accumulation of acids, primarily acetate. On the other hand, non-growing, aerated cell suspensions of the strain Zm6-*ndh*, with or without chloramphenicol addition, produced substantially less acids (0.57 g·L^{-1} acetate and 0–0.1 g·L^{-1} propionate); accordingly, their supernatants had a minor inhibitory effect upon *E. coli* (Table 2).

4. Discussion

Here, we present evidence that the key *Z. mobilis* antimicrobial compounds are acidic byproducts of its aerobic metabolism—acetate and propionate. Apparently, *Z. mobilis* does not require ribosomal protein biosynthesis for its inhibitory activity; thus, the antimicrobial compounds are not bacteriocins. Our data, therefore, do not support the conclusions of Lima et al. [19] that the antimicrobial compound of *Z. mobilis* might be a peptide. We found that the antimicrobial activity was generated by *Zymomonas* respiratory catabolism and was independent of cell growth and protein biosynthesis. Although *Z. mobilis* is known to produce ethanol at an extra high rate and tolerates ethanol concentration of more than 12% [2], which is detrimental for the majority of other bacteria (as it induces chaotropic effects and oxidative stress [30]), ethanol apparently is not its only and not even its key antimicrobial component. The *Z. mobilis* antimicrobial activity is much weaker under anaerobic conditions or in the non-respiring mutant, where ethanologenesis proceeds at its highest rate. High ethanol concentrations that are reached in stationary-phase *Z. mobilis* cultures when grown on sugar-rich media surely contribute to its antimicrobial effects

and may serve for outcompeting of other species in natural environments [31]. However, the *Z. mobilis* antimicrobial activity is also present in culture media with low ethanol concentration. Furthermore, we show that the inhibitory byproduct acetaldehyde, which accumulates in aerobic cultures and partially inhibits *Z. mobilis* growth, is not the major source of its antimicrobial activity against other species. It is the accumulation of acetate and propionate that is strongly associated with antimicrobial activity of either the culture supernatant or the extracellular medium of nongrowing, glucose-consuming cells.

Acetate and, in particular, propionate rank among the short-chain fatty acids having the strongest inhibitory effects on bacterial growth. Both are more potent inhibitors at lower pH. In the case of propionate, the synergy with low pH is particularly pronounced [32]. For *E. coli* growing at pH 6, the specific growth rate is reduced by approximately half in the presence of 8 mM acetate or 5 mM propionate [33]. Acetic acid sensitivity of the same order of magnitude has also been reported for the ESKAPE pathogens *Pseudomonas aeruginosa* [32] and *Staphylococcus aureus* [34]. In our experiments, acetate and propionate concentrations in the fermentation media of the aerobically grown strains Zm4 and Zm6 reached around 50 mM (3 $g \cdot L^{-1}$) and 30 mM (2 $g \cdot L^{-1}$), respectively (Figure 6). With 50% of added supernatant, the final concentrations of acetate and propionate in the growth inhibition experiments with *E. coli* and other bacteria were close to 25 and 15 mM, respectively; thus, they could account for the observed inhibitory effects, shown in Figure 2.

The acid resistance of *Z. mobilis* itself is higher. Growth of *Z. mobilis* Zm4 at pH 5 is almost unaffected by 8 $g \cdot L^{-1}$ (approx. 130 mM) acetate [35]. At pH 5.8, the IC_{50} of acetate for the recombinant strain *Z. mobilis* 8b is as high as 210 mM [20]. Since acetate is one of the main inhibitory components present in lignocellulose hydrolysates, much effort has recently been invested to further improve the acetate resistance in the recombinant *Z. mobilis* strains designed for lignocellulosic substrates [9,20,21]. The present work shows that the *Z. mobilis* resistance to acetate and propionate stress might also be relevant for its interaction with other microorganisms. Notably, while *Z. mobilis* is inhibitory for many microbial species, it can also coexist with a broad range of bacteria and yeasts [11]. At least some of those bacterial and fungal species have similar tolerance to or higher tolerance than acetic and/or propionic acid than *Z. mobilis*, e.g., some representatives of acetic acid bacteria and lactobacilli [36], for which the presence of *Zymomonas* is not inhibitory. We may speculate that the microorganisms associated with *Z. mobilis* in oxic natural habitats are able to tolerate the amounts of acetate and propionate that it generates, but further systematic study would be needed for concrete conclusions.

Metabolic pathways leading to the synthesis of propionate and acetate in *Z. mobilis* have not been elucidated in detail. The *Z. mobilis* genome does not carry a complete set of genes for the succinate, acrylate, 1,2-propanediol, or citramalate pathways of propionate production (for a review, see [37]). Involvement of amino-acid catabolic pathways in *Zymomonas* propionate synthesis remains a possibility to consider. Likewise, the genes encoding the dehydrogenase(s) involved in acetate synthesis, still need to be identified in this bacterium. Although, in the present work, we demonstrated dependence of the antimicrobial activity on the function of *Z. mobilis* aerobic respiratory chain and showed its association with production of acetate and propionate, we cannot completely exclude the involvement of additional, so far unidentified, low-molecular-weight inhibitory byproduct(s) of its respiratory catabolism. Therefore, deciphering the synthesis pathways of propionate and acetate, and constructing the respective knockout mutants would be the next steps in the study of the antimicrobial activity of *Z. mobilis*. Non-inhibitory *Zymomonas* strains or strains with controllable antimicrobial activity represent practical interest for the design of novel cocultivation bioprocesses and contribute to transforming this bacterium into a chassis microorganism for future metabolic engineering.

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References

1. Rogers, P.L.; Lee, K.J.; Skotnicki, M.L.; Tribe, D.E. Ethanol production by *Zymomonas mobilis*. In *Microbial Reactions. Advances in Biochemical Engineering*; Springer: Berlin/Heidelberg, Germany, 1982; Volume 23, pp. 37–84.
2. Sprenger, G. Carbohydrate metabolism in *Zymomonas mobilis*: A catabolic highway with some scenic routes. *FEMS Microbiol. Lett.* **1996**, *145*, 301–307. [CrossRef]
3. De Graaf, A.A.; Striegel, K.; Wittig, R.M.; Laufer, B.; Schmitz, G.; Wiechert, W. Metabolic state of *Zymomonas mobilis* in glucose-, fructose-, and xylose-fed continuous cultures as analysed by ^{13}C- and ^{31}P-NMR spectroscopy. *Arch. Microbiol.* **1999**, *171*, 371–385. [CrossRef] [PubMed]
4. Jacobson, T.B.; Adamczyk, P.A.; Stevenson, D.M.; Regner, M.; Ralph, J.; Reed, J.L.; Amador-Noguez, D. ^{2}H and ^{13}C metabolic flux analysis elucidates in vivo thermodynamics of the ED pathway in *Zymomonas mobilis*. *Metabol. Eng.* **2019**, *54*, 301–316. [CrossRef] [PubMed]
5. Kalnenieks, U.; de Graaf, A.A.; Bringer-Meyer, S.; Sahm, H. Oxidative phosphorylation in *Zymomonas mobilis*. *Arch. Microbiol.* **1993**, *160*, 74–79. [CrossRef]
6. Kalnenieks, U.; Balodite, E.; Rutkis, R. Metabolic engineering of bacterial respiration: High vs. low P/O and the case of *Zymomonas mobilis*. *Front. Bioeng. Biotechnol.* **2019**, *7*, 327. [CrossRef]
7. Rutkis, R.; Galinina, N.; Strazdina, I.; Kalnenieks, U. The inefficient aerobic energetics of *Zymomonas mobilis*: Identifying the bottleneck. *J. Basic Microbiol.* **2014**, *54*, 1090–1097. [CrossRef]
8. Pentjuss, A.; Odzina, I.; Kostromins, A.; Fell, D.A.; Stalidzans, E.; Kalnenieks, U. Biotechnological potential of respiring *Zymomonas mobilis*: A stoichiometric analysis of its central metabolism. *J. Biotechnol.* **2013**, *165*, 1–10. [CrossRef]
9. Wang, X.; He, Q.; Yang, Y.; Wang, J.; Haning, K.; Hu, Y.; Wu, B.; He, M.; Zhang, Y.; Bao, J.; et al. Advances and prospects in metabolic engineering of *Zymomonas mobilis*. *Metab. Eng.* **2018**, *50*, 57–73. [CrossRef]
10. Kalnenieks, U.; Pappas, K.M.; Bettenbrock, K. *Zymomonas mobilis* metabolism: Novel tools and targets for its rational engineering. *Adv. Microb. Physiol.* **2020**, *77*, 37–88. [PubMed]
11. Weir, P.M. The ecology of *Zymomonas*: A review. *Folia Microbiol.* **2016**, *61*, 385–392. [CrossRef]
12. Haffie, T.L.; Louie, P.W.; Khachatourians, G.G. Isolation of noninhibitory strains of *Zymomonas mobilis*. *Appl. Environ. Microbiol.* **1985**, *49*, 1007–1009. [CrossRef] [PubMed]
13. Souza, C.; Souza, L.A.G. Colpites and vulvovaginitis treatment using *Zymomonas mobilis* var. *recifensis*. *Rev. Inst. Antibiot.* **1973**, *13*, 85–87.
14. Swings, J.; De Ley, J. The biology of *Zymomonas*. *Bacteriol. Rev.* **1977**, *41*, 1–46. [CrossRef] [PubMed]
15. Santos, J.F.M.; Vasconcelos, J.; Souza, J.R.; Coutinho, E.M.; Montenegro, S.M.L.; Azevedo-Ximenes, E. The effect of *Zymomonas mobilis* culture on experimental *Shistosoma mansoni* infection. *Rev. Soc. Bras. Med. Trop.* **2004**, *37*, 502–504. [CrossRef] [PubMed]
16. Campos, I.A.; Ximenes, E.A.; Carvalho Júnior, C.H.R.; de Mesquita, A.R.C.; Silva, J.B.N.F.; Maia, M.B.S. *Zymomonas mobilis* culture protects against sepsis by modulating the inflammatory response, alleviating bacterial burden and suppressing splenocyte apoptosis. *Eur. J. Pharm. Sci.* **2013**, *48*, 1–8. [CrossRef] [PubMed]
17. Weuster-Botz, D.; Aivasidis, A.; Wandrey, C. Continuous ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part II: Process development for the fermentation of hydrolysed B-starch without sterilization. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 685–690. [CrossRef]
18. Fu, N.; Peiris, P.; Markham, J.; Bavor, J. A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/ xylose mixtures. *Enz. Microb. Technol.* **2009**, *45*, 210–217. [CrossRef]
19. Lima, G.M.S.; Souza, L.I.O.; Rosato, Y.B.; Araújo, J.M. Detection of bacteriocins in *Zymomonas mobilis* and RAPD fingerprinting of the producer strains. *Afr. J. Pharm. Pharmacol.* **2011**, *5*, 2132–2139. [CrossRef]
20. Franden, M.A.; Pilath, H.M.; Mohagheghi, A.; Pienkos, P.T.; Zhang, M. Inhibition of growth of *Zymomonas mobilis* by model compounds found in lignocellulosic hydrolysates. *Biotechnol. Biofuels* **2013**, *6*, 99. [CrossRef]

21. Yang, S.; Franden, M.A.; Brown, S.D.; Chou, Y.C.; Pienkos, P.T.; Zhang, M. Insights into acetate toxicity in *Zymomonas mobilis* 8b using different substrates. *Biotechnol. Biofuels* **2014**, *7*, 140. [CrossRef]
22. Kalnenieks, U.; Galinina, N.; Strazdina, I.; Kravale, Z.; Pickford, J.L.; Rutkis, R. NADH dehydrogenase deficiency results in low respiration rate and improved aerobic growth of *Zymomonas mobilis*. *Microbiology* **2008**, *154*, 989–994. [CrossRef] [PubMed]
23. Strazdina, I.; Kravale, Z.; Galinina, N.; Rutkis, R.; Poole, R.K.; Kalnenieks, U. Electron transport and oxidative stress in *Zymomonas mobilis* respiratory mutants. *Arch. Microbiol.* **2012**, *194*, 461–471. [CrossRef] [PubMed]
24. Kalnenieks, U.; Galinina, N.; Toma, M.M.; Pickford, J.L.; Rutkis, R.; Poole, R.K. Respiratory behaviour of a *Zymomonas mobilis* adhB::kanr mutant supports the hypothesis of two alcohol dehydrogenase isoenzymes catalysing opposite reactions. *FEBS Lett.* **2006**, *580*, 5084–5088. [CrossRef] [PubMed]
25. Markwell, M.A.; Haas, S.M.; Bieber, L.L.; Tolbert, N.E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206–210. [CrossRef]
26. Kalnenieks, U.; Balodite, E.; Strähler, S.; Strazdina, I.; Rex, J.; Pentjuss, A. Improvement of acetaldehyde production in *Zymomonas mobilis* by engineering of its aerobic metabolism. *Front. Microbiol.* **2019**, *10*, 2533. [CrossRef]
27. Wecker, M.S.; Zall, R.R. Production of acetaldehyde by *Zymomonas mobilis*. *Appl. Environ. Microbiol.* **1987**, *53*, 2815–2820. [CrossRef]
28. Yang, S.C.; Lin, C.H.; Sung, C.T.; Fang, J.Y. Antibacterial activities of bacteriocins: Application in foods and pharmaceuticals. *Front. Microbiol.* **2014**, *5*, 241. [CrossRef]
29. Yang, S.; Tschaplinski, T.J.; Engle, N.L.; Carroll, S.L.; Martin, S.L.; Davison, B.H. Transcriptomic and metabolomic profiling of *Zymomonas mobilis* during aerobic and anaerobic fermentations. *BMC Genom.* **2009**, *10*, 34. [CrossRef]
30. Cray, J.A.; Stevenson, A.; Ball, P.; Bankar, S.B.; Eleutherio, E.C.A.; Ezeji, T.C. Chaotropicity: A key factor in product tolerance of biofuel-producing microorganisms. *Curr. Opin. Biotechnol.* **2015**, *33*, 228–259. [CrossRef]
31. Cray, J.A.; Bell, A.N.W.; Bhaganna, P.; Mswaka, A.Y.; Timson, D.J.; Hallsworth, J.E. The biology of habitat dominance; can microbes behave as weeds? *Microb. Biotechnol.* **2013**, *6*, 453–492. [CrossRef]
32. Bushell, F.M.L.; Tonner, P.D.; Jabbari, S.; Schmid, A.K.; Lund, P.A. Synergistic Impacts of Organic Acids and pH on Growth of *Pseudomonas aeruginosa*: A Comparison of Parametric and Bayesian Non-parametric Methods to Model Growth. *Front. Microbiol.* **2019**, *9*, 3196. [CrossRef] [PubMed]
33. Roe, A.J.; O'Byrne, C.; McLaggan, D.; Booth, I.R. Inhibition of *Escherichia coli* growth by acetic acid: A problem with methionine biosynthesis and homocysteine toxicity. *Microbiology* **2002**, *148*, 2215–2222. [CrossRef] [PubMed]
34. Thomas, V.C.; Sadykov, M.R.; Chaudhari, S.S.; Jones, J.; Endres, J.L.; Widhelm, T.J. A Central Role for Carbon-Overflow Pathways in the Modulation of Bacterial Cell Death. *PLoS Pathog.* **2014**, *10*, e1004205. [CrossRef]
35. Joachimsthal, E.; Haggett, K.D.; Jang, J.H.; Rogers, P.L. A mutant of *Zymomonas mobilis* ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. *Biotechnol. Lett.* **1998**, *20*, 137–142. [CrossRef]
36. Lasko, D.R.; Zamboni, N.; Sauer, U. Bacterial response to acetate challenge: A comparison of tolerance among species. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 243–247. [CrossRef] [PubMed]
37. Gonzalez-Garcia, R.A.; McCubbin, T.; Navone, L.; Stowers, C.; Nielsen, L.K.; Marcellin, E. Microbial propionic acid production. *Fermentation* **2017**, *3*, 21. [CrossRef]