Zinkicide Is a ZnO-Based Nanoformulation with Bactericidal Activity against *Liberibacter crescens* in Batch Cultures and in Microfluidic Chambers Simulating Plant Vascular Systems

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ABSTRACT Phloem-limited bacterial “*Candidatus Liberibacter*” species are associated with incurable plant diseases worldwide. Antimicrobial treatments for these pathogens are challenging due to the difficulty of reaching the vascular tissue they occupy at bactericidal concentrations. Here, *in vitro* antimicrobial mechanisms of Zinkicide TMN110 (ZnK), a nonphytotoxic zinc oxide (ZnO)-based nanoformulation, were compared to those of bulk ZnO (b-ZnO) using as a model the only culturable species of the genus, *Liberibacter crescens*. Minimum bactericidal concentration (MBC) determination and time-kill assays showed that ZnK has a bactericidal effect against *L. crescens*, whereas b-ZnO is bacteriostatic. When ZnK was used at the MBC (150 ppm), its antimicrobial mechanisms included an increase in Zn solubility, generation of intracellular reactive oxygen species, lipid peroxidation, and cell membrane disruption; all of these were of greater intensity than those of b-ZnO. Inhibition of biofilms, which are important during insect vector colonization, was stronger by ZnK than by b-ZnO at concentrations between 2.5 and 10 ppm in batch cultures; however, neither ZnK nor b-ZnO removed *L. crescens* preformed biofilms when applied between 100 and 400 ppm. In microfluidic chambers simulating source-to-sink phloem movement, ZnK significantly outperformed b-ZnO in Zn mobilization and bactericidal activity against *L. crescens* planktonic cells in sink reservoirs. In microfluidic chamber assays assessing antibiofilm activity, ZnK displayed a significantly enhanced bactericidal activity against *L. crescens* individual attached cells as well as preformed biofilms compared to that of b-ZnO. The superior mobility and antimicrobial activity of ZnK in microenvironments make this formulation a promising product to control plant diseases caused by “*Candidatus Liberibacter*” species and other plant vascular pathogens.

IMPORTANCE “*Candidatus Liberibacter*” species are associated with incurable plant diseases that have caused billions of dollars of losses for United States and world agriculture. Chemical control of these pathogens is complicated, because their life cycle combines intracellular vascular stages in plant hosts with transmission by highly mobile insect vectors. To date, “*Candidatus Liberibacter*” species are mostly unculturable, except for *Liberibacter crescens*, a member of the genus that has been used as a model for *in vitro* assays. Here, we evaluated the potential of Zinkicide (ZnK) as an antimicrobial against “*Candidatus Liberibacter*” species in batch cultures

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and under flow conditions, using *L. crescens* as a biological model. ZnK displayed bactericidal activity against *L. crescens* in batch cultures and showed increased mobility and bactericidal activity in microfluidic devices resembling "*Candidatus Liberibacter*" species natural habitats. ZnK performance observed here against *L. crescens* makes this compound a promising candidate to control plant diseases caused by vascular pathogens.

KEYWORDS bactericidal, HLB, MBC, MIC, ZnO nanoparticle, biofilms, microfluidics, reactive oxygen species, solubilization, time-kill

Citrus greening, or Huanglongbing (HLB), is arguably the most devastating plant disease in citrus-producing areas worldwide (1). In the United States, HLB is associated with the phloem-limited bacterium "*Candidatus Liberibacter asiaticus*," which is vectored by the Asian citrus psyllid *Diaphorina citri*. This disease endangers the citrus industry in Florida and has already spread to other citrus-producing areas in the country, such as Texas and California (2). The control of HLB is especially difficult due to the intracellular nature of "*Candidatus Liberibacter asiaticus*" in its insect vector and plant hosts (3). The pathogen is transmitted by highly mobile vector insects that are difficult to contain, moving rapidly from initially infected trees to new areas (4). After feeding on infected plants, the pathogens inside their insect vector undergo a circulative transmission, where they grow planktonically and putatively form biofilms (5, 6). Upon further feeding on new plants, the bacteria are reinoculated into plant hosts from the insect salivary glands into the intracellular environment of the plant phloem (7).

The phloem-limited habitat of these pathogens makes conventional chemical control methods challenging or economically nonviable (1). The application of antibiotics has been explored with relative effectiveness (8–12), but this method is not cost-effective; applications need trained personnel to perform the activity and require trunk injections to deliver these antimicrobials directly into the plant phloem of each tree (9). Additionally, the use of antibiotics is also limited by the potential risk of increased microbial resistance in animal, plant, and human pathogens (9).

Nanoparticles have been extensively studied in drug delivery systems, offering a wide spectrum of formulations with improved physicochemical characteristics and curative properties (13, 14). Among them, zinc oxide (ZnO) nanoparticles represent an important class of commercially available materials and have been widely applied in diagnostics, therapeutics, drug delivery systems, and food preparation processes, among other fields (15).

The industry-grade formulation of Zinkicide (TMN110; here referred to as ZnK) is a novel antimicrobial composite that contains protein-size (~4.0-nm) ZnO nanoparticles coated with salicylic acid designed to control bacterial citrus diseases (16). The selection of Zn as the metal element of ZnK and the use of salicylic acid as a coat is supported by the preferential localization and transport of these two compounds by the phloem vasculature of citrus and other plant species (17–20). Preliminary studies demonstrated that a trunk-injectable 2S albumin-Nano-ZnO formulation achieved more than 95% reduction of "*Candidatus Liberibacter asiaticus*" populations without phytotoxic effects (21). On the other hand, ZnK nanoformulation has been shown to have a 2- to 8-fold lower MIC than traditionally used copper (Cu)-based compounds against *Xanthomonas citri* subsp. *citri*, *Escherichia coli*, and *X. alfalfae* subsp. *citrumelonis* (13). In field trials, ZnK displayed a significant reduction of citrus canker incidence after foliar sprays, showed lower phytotoxicity than commercially available copper-based compounds, and apparently displayed translaminar activity (13), which suggest a systemic movement of this novel formulation.

The unculturable nature of "*Candidatus Liberibacter*" species creates hurdles for studying the behavior of these pathogens *in vitro* (22). For this reason, several models have been used to understand the biology of these organisms (23–26). *Liberibacter crescens* strain BT-1 is the only culturable species and type strain of the *Liberibacter* genus (27), and it has the closest genomic context and synteny to "*Candidatus*

Liberibacter" species, as well as having similar associations with plant hosts (28). Thus, it represents the most accurate biological model available to study phenotypic features of "*Candidatus Liberibacter*" species (28). Working with *L. crescens* is a complex task that requires expensive rich media and long incubation periods for its growth (29). However, several culturing protocols for both planktonic (27, 29) and biofilm phases (5) that represent different stages of the "*Candidatus Liberibacter*" species life cycle have been reproducibly developed for their study *in vitro*.

In this work, we aimed to understand the *in vitro* antimicrobial activity and mode of action of ZnK against "*Candidatus Liberibacter*" species, using *L. crescens* strain BT-1 as a biological model. Our results showed that ZnK and bulk ZnO (b-ZnO) display similar antibacterial mechanisms, but such activities are significantly enhanced in the ZnK formulation, resulting in a bactericidal effect against *L. crescens*. We also confirmed that in microfluidic chambers that resemble "*Candidatus Liberibacter*" species natural habitats, ZnK mobilizes more than b-ZnO. The properties demonstrated *in vitro* for ZnK make this compound a promising product to control plant diseases caused by "*Candidatus Liberibacter*" species and other plant vascular pathogens.

RESULTS

Zinkicide has a bactericidal effect against *L. crescens*, while b-ZnO is bacteriostatic. Minimum inhibitory (MIC₅₀ and MIC₉₀) and bactericidal (MBC) concentrations were determined as previously described (30) for ZnK and b-ZnO in polystyrene 96-well plates using BM7 broth, in which *L. crescens* mostly grows in the planktonic fraction (5). MIC₅₀ was defined as the lowest ZnK or b-ZnO concentration that caused a reduction in the optical density at 600 nm (OD₆₀₀) of 50% or more compared to the untreated control culture turbidity after 7 days of incubation, while MIC₉₀ was defined similarly for a reduction in OD₆₀₀ of 90% or more under the same conditions. For b-ZnO, *L. crescens* OD₆₀₀ values after 7 days were significantly lower than those of the untreated control at concentrations of 25 ppm or more, and the MIC₅₀ threshold was achieved between 25 and 50 ppm. The *L. crescens* OD₆₀₀ reached a plateau at concentrations above 50 ppm but did not decrease below the threshold for MIC₉₀ for the concentration range tested here (Fig. 1A). On the other hand, for ZnK, the *L. crescens* OD₆₀₀ was significantly reduced at concentrations as low as 2.5 ppm and gradually decreased as ZnK concentration increased, reaching the MIC₅₀ between 10 and 25 ppm and the MIC₉₀ between 100 and 125 ppm (Fig. 1A). The MBC was defined as the lowest ZnK or b-ZnO concentration that caused a reduction in more than 99.9% in number of CFU/ml recovered compared to the population recovered from untreated control cultures. Considering the OD₆₀₀, the turbidity of *L. crescens* cultures treated with ZnK dropped below the initial OD (0.07) at 150 ppm, suggesting bacterial cell lysis. For ZnK-treated samples, MBC was determined at 150 ppm, achieving a reduction in culturable cell counts of almost 5 log₁₀ orders (>99.99) compared with the level of untreated samples. On the other hand, the criterion for MBC was not achieved for the highest b-ZnO concentration tested here, with a reduction of 2 log₁₀ orders (99.27%) attained at 200 ppm (Table 1).

Time-kill assays were consistent with MIC and MBC assays. The number of CFU/ml recovered from b-ZnO-treated samples after 24 h of exposure remained in the same order as the initial inoculum (~10⁴), consistent with a bacteriostatic mode of action. On the other hand, ZnK caused a reduction of 2 log₁₀ CFU/ml between 0 and 6 h and a more than 4 log₁₀ reduction at 12 h of exposure, supporting a bactericidal effect (Fig. 1B). This quantification established ZnK time-kill between 6 and 12 h when used at 150 ppm against 10⁴ CFU/ml of *L. crescens*.

Zinkicide solubilizes more Zn and induces higher oxidative stress levels than b-ZnO. Upon contact with aqueous media, solubilized Zn and reactive oxygen species (ROS) are released from ZnO-based nanoparticles, which are known to have a toxic effect against bacterial cells (31). For this reason, Zn solubilization and the production of ROS were assessed as toxicity mechanisms for b-ZnO and ZnK after 4 h of application. At the tested time no significant differences in total cell numbers or cell viability were observed between these two treatments (see Fig. S1 in the supplemental material);

FIG 1 MIC and time-kill assays. (A) Total growth measured as OD₆₀₀ of *Liberibacter crescens* cultures in BM7 exposed for 7 days to ZnK (black bars) or b-ZnO (gray bars). UTC, untreated control. Thresholds for MIC₅₀ and MIC₉₀ are marked in the chart as a thin and a thick dotted line, respectively. Different letters represent significant differences according to ANOVA Fisher's protected LSD test at a *P* value of <0.05 (*n* = 6) between different concentrations of the same treatment. Letters on top of each bar are assigned alphabetically from the highest to the lowest OD₆₀₀ value. (B) Number of CFU per milliliter for an untreated control or recovered at each time point after exposure to 150 ppm of ZnK or b-ZnO. Each point represents the average number of colonies recovered from 3 plate replicates. The dotted line marks a 3 log₁₀ reduction in number of CFU/ml compared with the initial number of CFU/ml used in the assay. A representative result from three independent experiments is shown here.

therefore, absolute values for each assay were directly compared. When 150 ppm of b-ZnO or ZnK was added to cell-free BM7 medium and incubated for 4 h, the Zn concentration in the supernatant of both treatments increased significantly compared to that of the untreated control (*P* < 0.05). However, the average soluble Zn concentration for ZnK-supplemented media was significantly higher than that for the media supplemented with b-ZnO (Fig. 2A). The use of the ROS indicator dihydroethidium revealed that both ZnK and b-ZnO (used at 150 ppm) triggered a significant increase of ROS in the presence of *L. crescens* cells compared to that of the untreated control, and ZnK caused significantly higher ROS production than b-ZnO (Fig. 2B).

Zinkicide induced a significant increase in DNA release and lipid peroxidation.

The presence of DNA and thiobarbituric acid species (TBARS) in the culture supernatant was measured as an indicator of membrane disruption and lipid peroxidation, respec-

TABLE 1 Minimum bactericidal concentration determination for b-ZnO and ZnK

Treatment	Culturable bacteria ^a (CFU/ml) at indicated treatment concn (ppm)			
	0	150	175	200
b-ZnO		$(2.14 \pm 0.13) \times 10^7$	$(1.41 \pm 0.20) \times 10^7$	$(5.28 \pm 0.13) \times 10^6$
ZnK		$(7.53 \pm 1.53) \times 10^3$	0	0
Untreated control	$(7.20 \pm 2.36) \times 10^8$			

^aNumbers of CFU/ml of *Liberibacter crescens* were quantified on BM7 agar plates for each treatment (ZnK, b-ZnO, and untreated control). *Liberibacter crescens* cells were exposed to treatments for 7 days and then plated, and colony counts were assessed after 5 weeks of incubation.

FIG 2 Antimicrobial mechanisms of b-ZnO and ZnK against *Liberibacter crescens*. (A) Zn solubilization in cell-free BM7 batch cultures at 4 h, as determined by inductively coupled plasma with optical emission spectroscopy (ICP-OES) ($n = 3$). (B) *L. crescens* intracellular reactive oxygen species (ROS) for each treatment determined by dihydroethidium (DHE) fluorescence at 4 h of exposure ($n = 6$). (C) DNA release from *L. crescens* cells for each treatment after 24 h of exposure, determined as the OD₂₆₀ of the supernatant normalized by total cell number quantified by qPCR at 24 h ($n = 3$). (D) Lipid peroxidation for each treatment at 24 h, determined as the OD₅₃₂ normalized by the total cell number quantified by qPCR at 24 h ($n = 3$). UTC, untreated control. Different letters on top of each bar represent significant differences between treatments according to ANOVA and Fisher's protected LSD test at a P value of <0.05 . Letters are assigned alphabetically from the highest to the lowest value on each chart. Error bars indicate standard deviations. The absence of error bars means that the error was too low to show. A representative result from more than two independent experiments is shown here.

tively. We chose 24 h for these assays, since it was the earliest time at which we observed differences in the number of viable cells detected by PMAxx-PCR for each treatment (Fig. S1B). Since ZnK decreased total cell numbers at 24 h to a greater extent than b-ZnO (Fig. S1A), we normalized the quantified signal for each technique with the total genomic DNA (genome equivalents) detected by quantitative PCR (qPCR) for each treatment. As observed in Fig. 2C, cultures treated with ZnK displayed the highest OD₂₆₀/total cell ratio, indicating they released a significantly larger amount of DNA per cell than the untreated controls and samples exposed to b-ZnO. Similarly, significant differences in normalized TBARS were observed for all treatments, among which the highest values were for *L. crescens* cultures supplemented with ZnK (Fig. 2D).

Zinkicide causes severe cell damage, visible by TEM. To visually confirm that ZnK was causing more severe cell damage than b-ZnO, *L. crescens* cultures treated for 24 h with either formulation (untreated control, b-ZnO, and ZnK) were observed by transmission electron microscopy (TEM). Untreated *L. crescens* cells were observed mostly in groups, showing round edges and actively dividing (Fig. 3A to C). On the other hand, despite also being observed in clusters, b-ZnO-treated cells displayed more angular and enlarged shapes (Fig. 3D to F). Finally, ZnK-treated cells were scarce, were rarely observed in clusters, and displayed severe cell shape deformations and damage that were interpreted as membrane disruption (Fig. 3G) and cell membrane outbursts (Fig. 3H), and, in most cases, cells were reduced to debris (Fig. 3I).

Zinkicide and b-ZnO inhibit *L. crescens* biofilm formation but do not disrupt *L. crescens* preformed biofilms. Both ZnK and b-ZnO inhibited *L. crescens* biofilm

FIG 3 Transmission electron microscopy (TEM) images of *Liberibacter crescens* cells left untreated or exposed for 24 h to 150 ppm of b-ZnO or ZnK. (A to C) Three examples of *L. crescens* cells observed in untreated samples. (D to F) Three examples of *L. crescens* cells treated with 150 ppm of b-ZnO. (G to I) Three examples of *L. crescens* cells treated with 150 ppm of ZnK. Scale bars: 1 μ m (A to C, G, and I) and 500 nm (D to F and H).

formation. However, ZnK biofilm inhibitory activity was more evident at lower concentrations than that for b-ZnO. In fact, b-ZnO significantly increased biofilm formation at 2.5 ppm and then gradually decreased at higher concentrations up to 125 ppm of b-ZnO, at which no further differences in inhibition were observed (Fig. 4A). For ZnK-treated samples, biofilm formation significantly decreased at concentrations as low as 2.5 ppm and gradually decreased at up to 100 ppm, and practically no further inhibition was observed for higher concentrations (Fig. 4B). Planktonic growth gradually decreased as b-ZnO and ZnK concentrations were increased in culture (Fig. 4A and B). On the other hand, *L. crescens* biofilms were not removed by b-ZnO or ZnK treatments in batch culture when applied at concentrations between 100 and 400 ppm (Fig. S2).

FIG 4 Biofilm formation inhibition assays. *Liberibacter crescens* cultures were inoculated at the initial time and assessed for planktonic growth and biofilm formation at 6 dpi. Each bar color represents the average value for biofilm and planktonic growth at each formulation concentration. (A) b-ZnO biofilm inhibition assays. (B) ZnK biofilm inhibition assays. Different letters represent significant differences between biofilm growth (black bars) or planktonic growth (gray bars) at different concentrations of b-ZnO and ZnK, according to ANOVA and Fisher's protected LSD test at P values of <0.05 ($n = 6$). Letters were assigned alphabetically from the highest to the lowest OD₆₀₀ value. A representative experiment from three independent experiments is shown here.

ZnK mobilizes more and kills more cells than b-ZnO in microfluidic chambers resembling a source-to-sink flow direction.

Mobility in microenvironments plays a critical role in the distribution of the two chemicals studied here in biological systems. Therefore, we used microfluidic chambers to compare ZnK and b-ZnO zinc mobility under flow conditions through microenvironments simulating plant vasculature (Fig. 5A and B). Our design consisted of a source syringe loaded with each treatment and a sink reservoir connected by a polydimethylsiloxane (PDMS) chip with 3 independent channels (80 μm wide, 3.8 cm long) (Fig. 5A and B). Treatments (MBC, $2\times$ MBC, and $3\times$ MBC in BM7 medium) were applied simultaneously at low constant flow speed (0.4 $\mu\text{l}/\text{min}$), and Zn concentration in culture media was quantified in sink reservoirs after 24 h. Since these experiments were intended to quantify mobilization in microenvironments under flow conditions, no centrifugation or filtration was applied to the collected effluents before element quantification by inductively coupled plasma with optical emission spectroscopy (ICP-OES). Zn concentration in sink reservoirs of ZnK-treated channels was significantly higher than that in sink reservoirs for b-ZnO-treated channels at all concentrations tested (Fig. 5C). No increase in Zn concentration was observed in sink reservoirs for b-ZnO-treated channels when applied at concentrations of 300 ppm or more, while Zn concentration in sink reservoirs for ZnK-treated channels gradually increased as ZnK concentration was increased (Fig. 5C). Bactericidal quantification assays in sink reservoirs showed that a differential Zn mobilization resulted in higher antimicrobial activity. Using the same experimental setup and after removing the 24-h initial treatment effluent, we inoculated an *L. crescens* bacterial suspension of 10^5

FIG 5 Zinc mobilization and bactericidal activity in sink reservoirs for b-ZnO and ZnK. (A) Image of the source-to-sink model system using microfluidic chambers. (B) Scheme of the microfluidic chamber design. (C) Zinc concentration in sink reservoirs at different concentrations of b-ZnO or ZnK inoculated in the source syringe. Different Latin letters represent significant differences in Zn concentrations between b-ZnO- and ZnK-treated channels applied at the same concentrations ($P < 0.01$ by Student's t test; $n = 3$). Different Greek letters represent significant differences in Zn concentrations in sink reservoirs between different concentrations of the same treatment (b-ZnO or ZnK) according to ANOVA and Fisher's protected LSD test at P values of < 0.01 ($n = 3$). (D) Bactericidal activity of b-ZnO and ZnK in sink reservoirs. Different Latin letters represent significant differences in average CFU/ml percentages between b-ZnO and ZnK applied at the same concentration ($P < 0.05$ by Student's t test; $n = 3$). Different Greek letters represent significant differences in average CFU/ml percentages between different concentrations of the same treatment according to ANOVA and Fisher's protected LSD test at P values of < 0.05 ($n = 3$). In panels C and D, letters were assigned alphabetically from the highest to the lowest value.

CFU/ml in BM7 medium in the sink reservoir, allowed it to receive the incoming treatment for 6 h, and later quantified the number of CFU/ml recovered from the resulting mix. As can be observed in Fig. 5D, no significant differences in the percentage of culturable bacterial population from sink reservoirs were observed between the different concentrations of b-ZnO inoculated from the source syringe. On the other hand, the proportion of CFU/ml recovered from ZnK treatments relative to the control value (provided as a percentage) gradually decreased as the ZnK concentration inoculated from the source syringe was increased, with significant differences between all the concentrations tested (Fig. 5D).

Zinkicide and b-ZnO inhibit *L. crescens* biofilm formation in microfluidic chambers. ZnK and b-ZnO biofilm inhibition activity was also assessed in microfluidic chambers. For this purpose, we quantified the surface area colonized by *L. crescens* biofilms during a 6-day period when exposed to a constant flow ($0.25 \mu\text{l}/\text{min}$) of inhibitory concentrations of b-ZnO (125 ppm), ZnK (125 ppm), and the nonsupplemented culture media. As can be observed in Fig. 6A, *L. crescens*-colonized surface area for b-ZnO- and ZnK-treated channels was maintained below $10 \mu\text{m}^2$ during the experiments, with no significant differences between treatments. On the other hand, the untreated channel displayed an exponential increase in surface area colonization after 72 h, with significant differences from b-ZnO- and ZnK-treated channels at 96 h ($P = 0.05$) and 144 h ($P = 0.02$).

FIG 6 Biofilm formation inhibition assessment for b-ZnO and ZnK in microfluidic chambers. (A) *Liberibacter crescens* surface area colonization quantification over time for the untreated control (UTC) and b-ZnO- and ZnK-treated channels. Points on each curve represent the average colonized surface area ($n = 3$) at each time point. Error bars show standard deviations. An asterisk represents significant differences in colonized surface area according to ANOVA and Fisher's protected LSD test ($P \leq 0.05$). (B) Bright-field images at $40\times$ for the UTC and b-ZnO- and ZnK-treated channels at different time points. Scale bar, $20\ \mu\text{m}$.

Zinkicide kills more *L. crescens* attached cells and preformed biofilms than b-ZnO in microfluidic chambers. To confirm that ZnK-improved Zn mobility led to enhanced bactericidal activity against adhered cells in microenvironments, we compared the ability of b-ZnO and ZnK to kill single *L. crescens* attached cells (at MIC_{90} , 125 ppm) and preformed biofilms (at $2\times$ MBC, 300 ppm) under flow conditions. For this purpose, we used the LIVE/DEAD BacLight bacterial viability kit inside the microfluidic chamber to reveal differences in cell viability for b-ZnO- and ZnK-treated channels. In biofilm inhibition assays, both dead and viable cells were observed in b-ZnO treatment (Fig. 7A). On the other hand, no viable cells were observed in any section of the ZnK-treated channels (Fig. 7B), which is consistent with its bactericidal mechanism. In biofilm disruption assays, as previously observed in batch culture systems (Fig. S2), neither b-ZnO nor ZnK disrupted *L. crescens* preformed biofilms (Fig. 8). However, a mix of viable and dead cells was observed in the b-ZnO-treated channel (Fig. 8A). On the other hand, no viable cells were observed in the ZnK-treated channels (Fig. 8B). To quantify these differences, we calculated the total area covered for live and dead cells separately and then calculated live/dead ratios by dividing the live area fraction by the dead area fraction. As can be observed in Table 2, average live/dead ratios in the ZnK-treated channels were significantly lower by several orders of magnitudes than those registered in b-ZnO-treated channels for biofilm inhibition ($P = 0.009$) and bio-

FIG 7 Cell viability assessment for b-ZnO and ZnK in microfluidic chambers for biofilm inhibition assays. The upper channel was inoculated with bBM7 + 0.75mβc medium (UTC, untreated control), and the lower channel was inoculated with the same medium supplemented with b-ZnO or ZnK at 125 ppm. (A and B) Bright-field and cell viability images at 40× for b-ZnO and ZnK treatments, respectively, at 6 dpi. Live cells are stained in green and dead cells are stained in red. Scale bar, 20 μm.

film disruption assays ($P < 0.001$). Viability differences observed in the selected sections of main channels were consistent all over the microfluidic device (Fig. S3 to S6).

DISCUSSION

In the present work, the antimicrobial activity of ZnK against *L. crescens* was assessed to understand possible mechanisms underlying the effect of this nanoformulation against “*Candidatus Liberibacter*” species. Results showed that b-ZnO and ZnK have similar effects against *L. crescens*. However, ZnK had bactericidal activity above the bacteriostatic effect of b-ZnO, possibly due to increased solubility, ROS formation, and cell lysis activity. Finally, our results suggest that the ZnK formulation is superior for moving in vascular tissues and, therefore, has higher probability to reach “*Candidatus Liberibacter*” species in the secluded environment of the phloem.

FIG 8 Biofilm disruption and cell viability assessment for b-ZnO and ZnK in microfluidic chambers. *L. crescens* was allowed to form biofilms for 6 to 8 days in microfluidic chambers. The upper channel then was inoculated with bBM7 + 0.75mβc medium (UTC, untreated control), and the lower channel was inoculated with the medium supplemented with b-ZnO or ZnK at 300 ppm for another 6 days. (A and B) Bright-field and cell viability images at 40× for b-ZnO and ZnK treatments, respectively, at 6 dpi. Live cells are stained in green and dead cells are stained in red. Scale bar, 20 μm.

TABLE 2 Live/dead ratios calculated for b-ZnO- and ZnK-treated channels in microfluidic chamber assays

Assay	Compared treatment	Live/dead (area ratio)		P value (Student t test)
		Avg	SD	
Biofilm inhibition	b-ZnO	1.78	0.65	0.009
	ZnK	4.45E−06	2.86E−06	
Biofilm disruption	b-ZnO	0.93	0.16	<0.001
	ZnK	7.18E−09	7.27E−09	

Experiments conducted here with ZnK showed MIC and MBC values against *L. crescens* similar to those determined for different metal nanoparticles against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio* spp., *Flavobacterium*, *Branchiophylum*, *Penicillium* spp., and *Mucor* spp. (32). *In vitro* assays with a previous reagent-grade formulation of ZnK, ZnK-SG6, showed a 2-fold lower MIC against *Xanthomonas citri* subsp. *citri*, *E. coli*, and *X. alfalfae* subsp. *citrumelonis* than the MIC of 125 ppm observed here for *L. crescens* and a 7-fold lower MIC than that for traditional Cu-based products (13). This difference may be because the SG6 formulation was made with reagent-grade chemicals (16) while the formulation of ZnK used here (TMN110) was made with industry-grade reagents. Additionally, antimicrobial performance can vary between different bacterial species and culture medium conditions (33, 34).

This study demonstrated that ZnK inhibited the growth of *L. crescens* at 125 ppm (MIC₉₀) and caused a 5 log₁₀ (CFU/ml) reduction in culturable cells at 150 ppm over the required 3 log₁₀ used to define the MBC of an antimicrobial compound (35). ZnK also caused a more than 4 log₁₀ reduction in culturability during the first 12 h when applied at the MBC. Both factors, the observed closeness between the MBC and the MIC and the strong culturability reduction induced in such a short time, confer a bactericidal mode of action for ZnK against *L. crescens*. On the other hand, the effect of b-ZnO to reduce *L. crescens* proliferation is limited, which classifies this compound as bacteriostatic.

Comparative assessment of antimicrobial mechanisms between b-ZnO and ZnK provided evidence to explain the bactericidal nature of the nanoformulation. Zinkicide displayed higher Zn solubilization in media, and, in the presence of *L. crescens*, increased ROS production compared to that of b-ZnO. In general, soluble Zn and ROS are released upon b-ZnO ionization in aqueous solution, and this process is strongly enhanced by the increase of surface contact with the solution that results from the reduction in particle size in ZnK (31).

ROS formation is considered the main antimicrobial mode of action of metal nanoparticles and is directly linked to ZnO ionization (31, 36, 37). In contact with water in solution, ZnO particles trigger a series of chemical reactions that produce peroxides (R–O–O–R), superoxide (O₂[−]), and hydrogen peroxide species (H₂O₂) (38, 39). The two first reactive species are cell impermeable and react at the cell membrane level, while H₂O₂ penetrates and acts intracellularly (40). The resulting change in intra- and extracellular oxidative status causes structural and functional cell damage that eventually leads to cell death (31). The increase in the amount of dissolved Zn in the culture medium containing bacteria is also an important toxicity mechanism described for ZnO nanoparticles (31, 41). Previous comparisons of CuO, NiO, ZnO, and Sb₂O₃ antimicrobial activity against several bacterial species found that the ZnO formulation displayed the highest solubilization rate and the second highest toxicity values (42). Li and collaborators also found that ZnO nanoparticle toxicity against *E. coli* was associated with free Zn ion release to the media and the concomitant formation of labile Zn complexes (43). Zinc is a very important micronutrient for living organisms that rely on its solubility for biological activity (44), being involved in almost every core biological process in the cell by binding in critical enzymes that mediate several catalytic reactions (45). However, when Zn reaches toxic levels it can rapidly react with several molecules and replace other weakly bound transition metals to metalloenzyme active sites, affecting several cellular processes (45).

Zinkicide caused cell lysis, as evidenced by severe cell shape deformation, disruption of cell membrane, and a quantifiable increase in the release of intracellular genetic material. Membrane disruption has also been described as an important antimicrobial mechanism by metal nanoparticles (31). TEM analysis has shown that ZnO nanoparticles can attach to the cell membrane, penetrate into the cell, and cause leakage of intracellular material in Gram-positive and Gram-negative bacterial species (31, 46, 47). Cell membranes can also be affected by the effect of free radicals released by ZnO nanoparticle dissolution in the media (48).

Superoxide (O_2^-) and hydroxyl radicals ($\bullet OH$) produced by metal nanoparticle ionization can react with cell membrane lipids, forming lipid radicals, aldehydes, and other reactive products that lead to structural membrane damage (49). Our results indicated that the application of both b-ZnO and ZnK indeed significantly increased the occurrence of lipid peroxidation, of which ZnK registered higher lipid peroxidation values. However, despite being statistically significant, such difference was minimal; therefore, lipid peroxidation may not be a major factor contributing to the differential antimicrobial activity observed between b-ZnO and ZnK.

Zinkicide was also effective in preventing *L. crescens* biofilm formation in both batch cultures and in microfluidic chambers, and we provided quantitative data demonstrating that ZnK is a more potent bactericide against sessile cells in biofilms than regular b-ZnO. Previous microscopic observations suggest that "*Candidatus Liberibacter*" species biofilm formation plays a role during the symbiosis process with their insect hosts (6, 50). The fact that "*Candidatus Liberibacter*" species undergo endocytosis in the insect gut (51) may require an adherent phase in the gut luminal phase. We speculate that since ZnK is expected to move systemically through the plant phloem, it could be ingested during insect feeding and act against initially adhered cells in the insect gut, decreasing cell viability, as shown in our assays in microfluidic chambers. However, ZnK did not remove *L. crescens* attached cells at concentrations as high as 300 ppm. Biofilms are well-known survival and resistance structures of prokaryotes that require stronger antimicrobial concentrations to be eradicated (52). Factors such as the sequestering of positive Zn ions by the negatively charged extracellular matrix (53), the insufficient antimicrobial compound diffusion inside the biofilm (54), and the metabolic heterogeneity of sessile cells can make biofilms more resilient to antimicrobial compounds (55).

The persistence of *L. crescens* biofilms in the presence of both b-ZnO and ZnK has precedents in other bacterial species. Similar responses that include increased cell surface adhesion and biofilm formation have been observed for the plant pathogen *Xylella fastidiosa* when exposed to Zn (56), Cu (57, 58), and calcium (Ca) (59). It is important to mention, however, that their influence in survival and virulence differs; Ca has been shown to enhance *X. fastidiosa* virulence traits *in vitro* (59), Cu increased virulence *in planta* (58), and Zn was shown to decrease pathogen proliferation and induce the formation of resilient biofilms *in vitro* (56).

Zinkicide was designed to display an enhanced mobility through plant phloem vessels (60), which "*Candidatus Liberibacter*" species inhabit with an irregular distribution (61). While b-ZnO has reduced solubility in water (4 ppm) (62) and a particle size in solution ranging from 300 to 900 nm, ZnK can hold an amount of 45,000 ppm of soluble metallic Zn in stock solution with an average particle size of 4 nm (16). Previous experiments under controlled conditions with ZnK-SG6 formulation in citrus trees already demonstrated that this compound has antimicrobial activity against *X. citri* subsp. *citri* cells inoculated into the leaf intercellular space (translaminar activity) (13). Here, with our assays in microfluidic chambers mimicking plant vascular systems, we also showed that ZnK has enhanced mobility in microfluidic environments compared to that of b-ZnO, resulting in a significantly higher accumulation and bactericidal activity in sink reservoirs. This mobilization ability also may allow ZnK to reach *L. crescens* attached single cells and preformed biofilms at a higher concentration than b-ZnO inside the microfluidic chamber. The additive effect of the differential antimicrobial activities at the same concentrations and mobilization capacities is what determined the observed differences in cell mortality under flow conditions. The increased accu-

mulation in sink reservoirs is also a highly desirable feature of this antimicrobial compound to control phloem-limited pathogens. This assay is a useful representation of the antimicrobial activity expected for ZnK in plant vascular systems and can be extended to other pathogen models and chemical compounds.

It is important to mention that further research is required to determine ZnK effective concentrations against "*Candidatus Liberibacter*" species *in vivo*. Parameters such as ZnK penetration into the plant, accumulation in the phloem, and clearance time should be addressed in future studies for an effective application of ZnK as a chemical control of HLB. The reduced genome size of "*Candidatus Liberibacter*" species compared to that of *L. crescens* (63) and the lack of genes for a free-living style in these pathogens, especially those to cope with oxidative stress (64), suggest that a higher antimicrobial activity can be achieved by ZnK against "*Candidatus Liberibacter*" species *in vitro*. However, "*Candidatus Liberibacter*" species persistence in their natural habitats is determined by their close relationships with their plant and insect host intracellular environments (4, 5) and by the accompanying microbiota (65, 66). Zinc and ROS homeostasis mechanisms in plants (67, 68), insects (69, 70), and cohabiting bacterial species (71, 72) may modulate "*Candidatus Liberibacter*" species survival after ZnK applications in nature. Therefore, future work in greenhouses and field trials addressing "*Candidatus Liberibacter*" species complex interactions with their habitat will further inform on ZnK efficacy against plant diseases caused by these pathogens.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. crescens* strain BT-1 was grown on BM7 agar plates (27) at 28°C. Stocks of *L. crescens* cultures were stored in BM7 broth plus 20% glycerol at –80°C. *L. crescens* cultures were started from cryovials and allowed to grow until slight bacterial growth was observed (5 to 6 days). A loopful of initially grown *L. crescens* cells was transferred to a fresh BM7 agar plate, allowed to grow for three to four additional days, and subsequently used for cell suspension preparation for downstream assays. *L. crescens* cultures in BM7 broth were used for MIC and MBC determination, time-kill assays, DNA release, and lipid peroxidation and ROS quantification, while *L. crescens* cultures in bBM7 + 0.75mβc broth (5) were used for biofilm assays in batch cultures and microfluidic chambers.

Zinkicide and bulk ZnO formulations. The commercial-grade formulation of Zinkicide TMN110 (4.5% metallic Zn; ~4-nm particle size) (TradeMark Nitrogen, Tampa, FL, USA), referred to here as ZnK, was used as an antimicrobial ZnO nanoformulation, and bulk ZnO (b-ZnO) (300- to 900-nm particle size) (Zinc Oxide, LLC, Dickson, TN, USA) was used as non-nanoformulated ZnO control.

ZnK TMN110 is an industry-grade liquid bactericide formulation manufactured and supplied as a gift by TradeMark Nitrogen, Inc. (Tampa, FL, USA), that is based on the patented Zinkicide SG6 chemical-grade formulation (16). Synthesis and composition of SG6 are briefly described here. The overall method of preparing the Zinkicide SG6 formulation includes mixing a water-soluble Zn source, a surface-capping agent, and an oxidizing agent. Briefly, in a glass beaker, 40 ml deionized water was mixed with 10 ml of 30% hydrogen peroxide (oxidizing agent), 2.5 ml sodium salicylate solution (32.8% [wt/wt]) (surface-capping agent), and 5 ml Zn nitrate stock solution (59% [wt/wt]) (water-soluble zinc source). The mix was magnetically stirred overnight, and then the pH was adjusted to 7.5 with 1 N NaOH (approximately 25 ml) (16). The resulting nanoparticle is formed by a ZnO core and a coat of salicylic acid. In ZnK, a combination of weak organic acids was used to stabilize H₂O₂, which becomes a controlled source of nascent oxygen to convert Zn(OH)₂ to nano-ZnO. The molar ratio of H₂O₂ to acid (carboxyl group) was 1:1.2. The detailed composition and the process of making industry-grade ZnK (TMN110) is proprietary information of TradeMark Nitrogen, Inc. (Tampa, FL, USA).

The bulk ZnO (b-ZnO) formulation was prepared as follows. Five hundred milligrams of ZnO (Zinc Oxide, LLC, Dickson, TN, USA) was added to a glass flask and rinsed to a total volume of 10 ml to reach a final concentration of 40,000 ppm. The tube then was vigorously vortexed and the solution was sonicated for 3 min to disperse ZnO particles. The flask then was wrapped with aluminum foil and sterilized in an autoclave at 121°C/1 atm for 15 min. Vigorous vortexing for several minutes was applied every time b-ZnO was used to ensure homogeneous particle dispersion. Both ZnK and b-ZnO stock formulations were stored at 4°C.

MIC and MBC determination. MIC and MBC assays were performed in polystyrene 96-well plates (COSTAR, Kennebunk, ME, USA) as previously described (35), with some modifications. To avoid desiccation, all wells in column number 1 and rows A and H were filled with 250 μl of sterile deionized water (dH₂O), while 260 μl dH₂O was used for column number 12. Ten microliters of an *L. crescens* bacterial suspension in BM7 (OD₆₀₀ of 1.0) was inoculated into 190 μl of BM7 (final OD₆₀₀ of 0.07) [(3.27 ± 0.27) × 10⁵ CFU/ml] and supplemented with ZnK and b-ZnO at final concentrations of 0, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125, 150, 175, and 200 ppm. Both ZnK and b-ZnO solutions in main stocks and in culture media were vigorously agitated before dispensing to ensure accurate final concentrations on each treatment solution and on each replicate in 96-well plates. This was particularly important because b-ZnO precipitates at concentrations higher than 100 ppm. Precipitation was not observed with ZnK at the concentrations used here, but the same protocol was used to ensure comparable treatments. Six blanks

and six replicates (wells) were included for each treatment. OD₆₀₀ measurements for all treatment samples were obtained from the difference of the cell-inoculated wells and its corresponding blank well with the exact same concentration of b-ZnO or ZnK. Plates were wrapped with one parafilm layer, reinforced with one layer of surgical tape (Durapore; 3M, St. Paul, MN, USA), and incubated at 28°C, 140 rpm, for 7 days. Constant agitation was used to allow precipitated particles of b-ZnO to contact all cells in the wells. At the end of the incubation period, the OD₆₀₀ of each well was read using a Cytation 3 image reader spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). Significant differences in OD₆₀₀ among treatments were determined using analysis of variance (ANOVA) and Fisher's least significant difference (LSD) method at a significance level of <0.05 using Sigma Plot software (version 11.0). The MIC₅₀ was defined as the lowest ZnK or b-ZnO concentration that caused a reduction in the OD₆₀₀ in 50% or more of the untreated control culture turbidity after 7 days of incubation, while the MIC₉₀ was defined as the lowest ZnK or b-ZnO concentration that caused a reduction in the OD₆₀₀ in 90% or more of the untreated control culture turbidity after 7 days of incubation (30). These same plates were used for MBC determination. Briefly, after the 7-day incubation period, 3 wells per treatment were agitated by pipetting, and 50 µl of each well replicate for each concentration of ZnK and b-ZnO from 125 to 200 ppm and the untreated control was plated in BM7 agar plates using an Eddy Jet 2 spiral plate spreader (Torrent de l'Estadella, Barcelona, Spain) by following a logarithmic dilution protocol (73). Plates were incubated at 28°C for 5 weeks. To counteract desiccation effects during the long incubation period, plate stacks were placed in plate bags together with an extra plate filled with sterile deionized water and wrapped with parafilm. The MBC was determined as the compound concentration where colony recovery was decreased more than 99.9% compared with the untreated control (30). Experiments were repeated three times independently.

Time-kill assay. The time-kill assay was performed as previously described (35). Briefly, an *L. crescens* culture in early exponential phase was adjusted to ~10⁴ CFU/ml in BM7 medium and divided into nine 5-ml aliquots. Aliquots were inoculated by triplicate with 150 ppm of either b-ZnO or ZnK or remained untreated (control). Tubes were incubated at 28°C, 140 rpm. Every 6 h for a period of 24 h, 100 µl of the sample and the -1 dilution of each replicate were streaked on BM7 agar plates and incubated at 28°C. Colonies formed were quantified between 3 and 5 weeks after plating. The time at which a reduction of 3 log₁₀ in the number of CFU/ml was achieved compared with that recovered from the untreated control at time zero was considered the time-kill. Experiments were repeated three times independently.

Total and viable cell quantification using PMAxx-qPCR. Five-milliliter aliquots of *L. crescens* cultures (OD₆₀₀ of 0.6; ~10¹⁰ total cells) in 50-ml Falcon tubes were inoculated by triplicate with 150 ppm of either b-ZnO or ZnK, and a noninoculated treatment was used as an untreated control. Each treatment was set in triplicate and incubated at 28°C and 140 rpm. Two 200-µl aliquots then were collected from each treatment replicate at 0, 24, and 48 h. One of the aliquots collected at each time point was treated with 0.5 µl of 20 mM PMAxx (Biotium, Inc., Fremont, CA, USA) according to the manufacturer's instructions (final PMAxx concentration of 50 µM). DNA extraction was performed using a modified CTAB protocol (74). The primers Lcr-F (5'-TAAAGATAGTGCTAAACAGGT-3') and Lcr-R (5'-TGATGACTCCCTTTT ACCAA-3') and the probe Lcr-P (5'-FAM-TCGCCATTAGCAGAAATCGTT-TAMRA-3') were designed using OLIGO v7 software (Molecular Biology Insights, Colorado Springs, CO, USA) (75) to amplify a 134-bp fragment from a conserved region of the gene that codes for the subunit B of *L. crescens* RNA polymerase (*rpoB*) and assessed for specificity using the Primer-BLAST tool of NCBI (76). A four-point standard curve consisting of DNA extracted from 10-fold serial dilutions of *L. crescens* bacterial suspensions was coamplified with the samples for absolute genome equivalent quantification. Aliquots of standards were used twice before disposing. Amplification reactions were performed using a C1000 Touch thermal cycler model CFX96 real-time system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The amplification parameters used were 95°C for 2 min, followed by 39 cycles of 95°C for 5 s and 60°C for 30 s. Three biological and three technical replicates were included for each time sampled.

Release of intracellular content and lipid peroxidation quantification. The leakage of intracellular content was quantified at 24 h as DNA release by spectrophotometry of supernatants at 260 nm (48). Briefly, 5-ml *L. crescens* cultures (OD₆₀₀ of 0.6) were exposed to 150 ppm of b-ZnO or ZnK for 24 h. After this time, 2-ml aliquots were transferred to 2-ml Eppendorf tubes and centrifuged at 5,000 rpm for 10 min. Aliquots (1 ml) of the supernatant of each tube then were removed with a 1-ml plastic syringe without disturbing the cell pellet and filtered with a 0.2-µm polytetrafluoroethylene (PTFE) syringe filter (VWR). The absorbance of each filtrate was measured at 260 nm using a Cytation 3 image reader spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Absorbance values obtained at 260 nm were normalized by division by the total number of genome equivalents obtained at 24 h by qPCR. Lipid peroxidation was also determined after 24 h of exposure by spectrophotometric TBARS quantification as previously described (80) and normalized to the number of genome equivalents. Three replicates were included per treatment, and the experiments were repeated twice.

Microscopic morphological changes of *L. crescens* in response to ZnK and b-ZnO assessed by TEM. *L. crescens* bacterial suspensions (OD₆₀₀ of 0.6) in BM7 and in BM7 supplemented separately with 150 ppm of ZnK or b-ZnO were incubated for 24 h at 28°C and 140 rpm. Cell-free BM7 broth supplemented separately with 150 ppm of ZnK and b-ZnO was included as a control. After the incubation period, 5 µl of each sample was added to Formvar-carbon-coated 300-mesh Cu support film grids (Electron Microscope Sciences, Hatfield, PA, USA) and allowed to settle for 2 h. At this time, 5 µl of phosphotungstic acid (Matheson, Coleman & Bell, Norwood, OH, USA) was added to each grid and carefully removed after 20 s with a filter paper. Grids were air dried and used for observation and image capturing using a Zeiss EM 10 transmission electron microscope (Carl Zeiss) controlled by MaxIm DL software (Diffraction Ltd., Ottawa, ON, Canada).

Zinc solubility determination in batch systems. Soluble Zn content on each treatment was determined as previously described (77). Briefly, 5 ml of BM7 liquid medium was supplemented with 150 ppm of b-ZnO or ZnK separately and incubated for 4 h at 28°C and 140 rpm. After this time, 2-ml aliquots were transferred to 2-ml Eppendorf tubes and centrifuged at 10,000 rpm for 10 min, and then 1 ml of the supernatant of each tube was removed with a 1-ml plastic syringe and filtered with a 0.2- μ m PTFE syringe filter (VWR). Every Zn particle that was filtered through the 0.2- μ m membrane (smaller than 200 nm) was assumed to be soluble Zn (77). Next, 10 μ l of each sample was collected and diluted in 900 μ l of molecular-grade water. Finally, Zn concentration was quantified by ICP-OES as previously described (78). Three replicates were included per treatment, and experiments were repeated three times.

ROS quantification. *L. crescens* ROS production in response to ZnK and b-ZnO was determined using dihydroethidium (Life Science Technologies, Eugene, OR, USA). Six-day-old *L. crescens* cells in BM7 agar plates were scraped and suspended in prewarmed (28°C) phosphate-buffered saline (PBS) to a final OD₆₀₀ of 1.0. The dye then was added to reach a final concentration of 10 μ M, and the tube was wrapped in foil and incubated in the dark for 15 min at 28°C and 140 rpm. *L. crescens* cells then were centrifuged at 5,000 rpm for 10 min, the excess dye was removed with the buffer, and the cells were washed twice with prewarmed PBS and finally resuspended in the same volume of BM7. The cell suspension with the internalized dye then was split into three equal volumes. Two of them were supplemented with 150 ppm of ZnK or b-ZnO, respectively, and one remained untreated. Finally, the volume of each treatment and the untreated control were dispensed in a six-well column of a 96-well plate for measurement. Fluorescence intensity was read with excitation/emission wavelengths of 518 and 606 nm, respectively, using a Cytation 3 image reader spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at the initial time point and every hour for 4 h. Significant differences between average ROS production were determined by ANOVA and Fisher's LSD statistical test as described above. Experiments were repeated twice. One representative replicate is shown here.

Biofilm formation inhibition assays in batch cultures. For biofilm formation inhibition, bBM7 + 0.75m β c medium was used for *L. crescens* bacterial suspension preparation and biofilm formation in 96-well plates, as previously described (5). Treatments were inoculated and incubated as described above for MIC determination. Planktonic growth was determined by OD₆₀₀ readings at 6 days postinoculation (dpi), while biofilm formation inhibition was assessed as previously described (5), with the modification of reducing water washing steps to two. Significant differences in turbidity readings between the planktonic and biofilm growth for each treatment were calculated by ANOVA and Fisher's LSD method as described above.

Biofilm disruption assays in batch cultures. For biofilm disruption assays, *L. crescens* bacterial suspensions were inoculated in polystyrene 96-well plates and incubated for 6 days as described above, but no treatments were added to the cultures to allow biofilm formation. After the initial incubation time, the liquid of each well was gently removed, including blanks with a multichannel pipette, refilled with fresh treatments of ZnK and b-ZnO at concentrations of 0, 100, 200, 300, and 400 ppm, and incubated under the same conditions for 6 additional days. Planktonic and biofilm growth was quantified as described above after the second incubation period.

Biofilm formation inhibition and biofilm disruption assays in microfluidic chambers. Microfluidic chamber design and fabrication were performed as previously described (79). For biofilm formation inhibition assays, the upper channel was initially filled with bBM7 + 0.75m β c broth and bBM7 + 0.75m β c broth supplemented with Zinkicide or b-ZnO at 125 ppm in the lower channel using an automated syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA, USA). *L. crescens* bacterial suspensions in bBM7 + 0.75m β c medium (OD₆₀₀ of 1.0) were injected until ~5 attached cells/20 μ m² were observed. The bacterial culture inlets then were clamped with surgical scissors, and the flow rate was maintained at 0.25 μ l/min for 6 days. For biofilm disruption assays, both channels were initially filled with bBM7 + 0.75m β c broth, and *L. crescens* cells were inoculated as described above and allowed to form biofilm for 6 to 7 days. After this time, the culture medium in one of the channels was replaced by bBM7 + 0.75m β c broth supplemented with 300 ppm of ZnK or b-ZnO and allowed to flow at the same rate for 6 additional days. Microfluidic chambers were mounted onto a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY, USA) and observed with a 40 $\times$ objective using phase contrast and Nomarski differential interference contrast (DIC) optics. Cell proliferation over time was recorded using time-lapse microscopy. Image acquisition was performed automatically every 10 min using a Nikon DS-Q1 digital camera (Nikon, Melville, NY, USA) controlled by NIS-Elements software, version 3.2 (Nikon, Melville, NY, USA). At the endpoint of the experiments, *L. crescens* cell viability inside the microfluidic chamber was assessed using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Eugene, OR, USA). Briefly, 1.5 μ l of each reagent of the kit was added to 500 μ l of the same treatments running in the microfluidic chamber, loaded into a 1-ml plastic syringe (BD Becton, Dickinson, UT, USA), aseptically interchanged by the treatment running in the microfluidic chamber, and inoculated in the dark at the same flow rate as that used above for 18 to 24 h. To detect both dyes used, propidium iodide and SYTO 9, excitation wavelengths of 528 nm (fluorescein isothiocyanate [FITC]) and 590 nm (tetramethyl rhodamine isothiocyanate [TRITC]), respectively, were used. Image capture and analysis were performed using a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY, USA) fitted with a Nikon DS-Q1 digital camera (Nikon, Melville, NY, USA) and controlled by NIS-Elements software, version 3.2 (Nikon, Melville, NY, USA). At least three independent experiments were performed for each treatment.

Mobilization and bactericidal activity of b-ZnO and ZnK in sink reservoirs of microfluidic chambers. To assess Zn mobilization in sink reservoirs of microfluidic chambers, 5 ml of BM7 was supplemented with concentrations of 150, 300, and 450 ppm of ZnK or b-ZnO and loaded into 5-ml plastic syringes.

The microfluidic chamber was primed with the nonsupplemented medium at 40 $\mu\text{L}/\text{min}$ for 30 min, and then the source syringes were replaced with the aforementioned treatments and the flow was maintained at 0.4 $\mu\text{L}/\text{min}$. Twenty-four hours later, the effluent of the collection syringes was aseptically removed with a 1-ml pipette, and the collection syringes were aseptically replaced by new syringes. Four hours later, 10 μL of the effluent was collected and diluted in 900 μL of molecular-grade water. Zn concentration for each treatment was quantified by ICP-OES as previously described (78). Bactericidal activity in sink reservoirs was quantified using the same experimental setup with some additional steps in independent experiments. Following the effluent collection from sink syringes after the initial 24 h of treatment pumping, 200 μL of an *L. crescens* bacterial suspension in BM7 medium with 10^5 CFU/ml was aseptically inoculated in sink syringes that were capped with sterile cotton plugs. Syringe pumps were not stopped during this operation, so treatments continued flowing at 0.4 $\mu\text{L}/\text{min}$. Six hours later, the volume of every sink reservoir (including inoculated cells and the flowed treatment during the 6-h period) was aseptically homogenized with a 200- μL pipette, and 100 μL of the volume was aseptically transferred to a sterile 0.2-ml Eppendorf tube. The numbers of CFU recovered from sink reservoirs of every treatment, including untreated controls, were determined as previously described using an Eddy Jet 2 spiral plate spreader (Torrent de l'Estadella, Barcelona, Spain) by following a logarithmic dilution protocol (73). Changes in the number of CFU/ml were represented as percentages of the untreated controls included in each experiment. Significant differences in average CFU/ml percentages between different concentrations of the same formulation were determined by ANOVA and Fisher's LSD. Significant differences in average CFU/ml percentages between different formulations applied at the same concentration were calculated by Student's *t* test. All statistical analyses were performed at a significance level of <0.05 .

Surface area colonization and live/dead ratio quantification in microfluidic chambers. Colonized surface area for bright-field time-lapse experiments, and total area covered by live and dead fluorescent cells in b-ZnO- and ZnK-treated channels, were quantified using the Object Count tool of the software NIS-Elements 3.2 Advanced Research (Nikon, Melville, NY, USA). Briefly, after activating the region of interest (ROI) setting in the Measurement menu, a rectangular ROI was drawn along the center of the channel, covering one-third of the total vertical area of the channel. This method was used to avoid bias due to changes in shear stress in channel edges. The region of the entire image (inside or outside the ROI) with the darkest spot (for bright-field captures) or the highest fluorescence intensity (for FITC or TRITC channels) then was selected with the Object Count selection tool, and then threshold settings were manually adjusted until object limits detected by the software fit with the actual object limits in the original image. Recorded object counts for each frame and their respective areas then were exported in an Excel file. The total area obtained in each frame for each channel (bright field, FITC for live cells, or TRITC for dead cells) was calculated by summing the area of all the independent objects detected in the ROI. Main sections of three independent experiments were used for all the assays. For fluorescence capture, each channel (FITC for live cells and TRITC for dead cells) was quantified separately. After performing this task for viability images in both FITC and TRITC channels, live/dead ratios for each treatment replicate were calculated by dividing the total area detected in the ROI with the FITC channel by total area detected in the ROI with the TRITC channel. In cases where no objects were present for the FITC channel, the area was arbitrarily set to 0.001 μm^2 . Significant differences between live/dead ratios obtained in b-ZnO- and ZnK-treated channels were determined using a Student's *t* test at a significance level of <0.05 .

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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