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Loss of Gramicidin Biosynthesis in Gram-Positive Biocontrol Bacterium *Aneurinibacillus migulanus* (Takagi et al., 1993) Shida et al. 1996 Emend Heyndrickx et al., 1997 Nagano Impairs Its Biological Control Ability of *Phytophthora*

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Abstract: The soil-borne species *Aneurinibacillus migulanus (A. migulanus)* strains Nagano and NCTC 7096 were shown to be potent biocontrol agents active against several plant diseases in agricultural and forest ecosystems. Both strains produce the cyclic peptide gramicidin S (GS) that was described as the main weapon inhibiting some gram-negative and gram-positive bacteria and fungus-like organisms along with the production of biosurfactant and hemolysis activities. However, the contribution of the cyclic peptide gramicidin S (GS) to the biocontrol ability of *A. migulanus* has never been studied experimentally. In this paper, using a mutant of the *A. migulanus* Nagano strain (E1 mutant) impaired in GS biosynthesis we evaluated the contribution of GS in the biocontrol potential of *A. migulanus* against *Phytophthora* spp. The two strains of *A. migulanus*, Nagano and NCTC 7096, were tested in a pilot study for the inhibition of the growth of 13 *Phytophthora* species in dual culture assays. *A. migulanus* Nagano was significantly more inhibitory than NCTC 7096 to all species. Additionally, using apple infection assays, *P. rosacearum* MKDF-148 and *P. cryptogea* E2 were shown to be the most aggressive on apple fruits displaying clear infection halos. Therefore, the three *A. migulanus* strains, Nagano, NCTC 7096, and E1, were used in apple infection experiments to check their effect on infection ability of these two *Phytophthora* species. Treatment with *A. migulanus* Nagano significantly reduced the severity of symptoms in apple fruits compared with NCTC 7096. *A. migulanus* E1 mutant showed total loss of biocontrol ability suggesting that GS is a major actor in the biocontrol ability of *A. migulanus* Nagano strain.

Keywords: secondary metabolism; bioinformatics; genome mining; *Aneurinibacillus migulanus*; biocontrol bacteria; gramicidin S; biosurfactant

1. Introduction

The biocontrol of plant diseases in agricultural and forest ecosystems was recently favored over other means to cope with plant pathogens [1–7]. Environmental [3,7], multitactic [4,6,8], and economic reasons were always acknowledged for using biocontrol versus

chemical control [9–13]. Biological control agents act via a range of modes of action including (1) induced resistance and priming of the host plant [14], (2) indirect interaction with pathogens and competition with plant pathogens [15], and (3) direct interaction with pathogens mainly through hyperparasitism [16] and antibiosis by antimicrobial metabolites [13,17,18] among other modes of action and the mixed modes of action [19–21].

The soil-borne species *A. migulanus* strains Nagano and NCTC 7096 were shown to be potent biocontrol agents against several plant diseases in agricultural and forest ecosystems. Alenezi et al. [22] showed the efficiency of *A. migulanus* strains Nagano and NCTC 7096 in the control of forest trees' pathogens such as *Dothistroma septosporum.* Both strains produce the cyclic peptide gramicidin S (GS) that was described as the main weapon inhibiting some gram-negative and gram-positive bacteria and fungus-like organisms along with the production of biosurfactant and hemolysis activities [17,22–24]. Gramicidin S (GS) is a cationic cyclic decapeptide with the primary structure [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] active against many gram-positive and some gram-negative bacteria [17]. Gramicidins act through forming small pores in the cell membrane [17]. The full genome sequence of both strains, *A. migulanus* Nagano and NCTC 7096, was determined [25,26].

GS-deficient mutants in *A. migulanus* were reported [27]. Five different mutant classes were discovered including the E1 mutant that is unable to synthetize GS or d-phenylalanyl-l-propyl diketopiperazine (DKP). The contribution of gramicidins to the biocontrol ability in *A. migulanus* have never been investigated. In this manuscript and using *A. migulanus* Nagano *and* NCTC 7096 as well as *A. migulanus* Nagano mutant E1 we investigated the contribution of gramicidins to the biocontrol ability of *A. migulanus*. Our results unambiguously documented that gramicidins are major actors of the biocontrol ability of *A. migulanus*.

2. Materials and Methods

2.1. Growth Conditions for Bacteria

A. migulanus Nagano (including the E1 mutant) and NCTC 7096 strains were obtained from the Institute of Biological and Environmental Sciences' (University of Aberdeen, Aberdeen, UK) culture collection and the National Collection of Type Cultures (NCTC, Porton Down, Salisbury, UK), respectively. The different strains were cultured on tryptone soya agar media following the close recommendations of Alenezi et al. [17]. Growth curves of bacteria and enumeration were also carried out according to Alenezi et al. [17].

2.2. Growth Conditions for Phytophthora Species

All *Phytophthora* species used in the present study are described in Table 1. All strains were maintained on potato dextrose agar (PDA; Oxoid, Basingstoke, Hants, UK) for 10 days at 25 °C. All cultures were routinely subcultured at 15-day intervals.

2.3. Screening for Biosurfactant Activity Using Surface Tension Method

Surface tension measurements were conducted according to Alenezi et al. [17]. Briefly, 16-hour bacterial suspension cultures of *A. migulanus* Nagano or NCTC 7096 strains (4 mL) were mixed with 400 mL of TSB in 1-L conical flasks. Flasks were then incubated at 37 °C with continuous shaking at 180 rpm for 48 h. Then 1 mL of the resulting cultures was centrifuged (10,000× *g* for 2 min) and the supernatant was collected. The resulting cell-free supernatant was transferred to a 5-cm-diameter concave glass dish. After 5 min of incubation, a thin ring was immersed and pulled out of the sample. The maximum force necessary to remove the ring was recorded and expressed in mNm^{-1}. Negative controls were carried out using distilled water and TSB. At least five replicates were conducted for each measurement.

Table 1. Sources of *Phytophthora* species used in dual culture antagonism tests with *A. migulanus*.

Clade	*Phytophthora* Species	Strain	Origin
1	*P. cactorum*	P-138	Kent
2	*P. plurivora* *P. citrophthora*	MKDF-179 P-139	Macedonia Kent
3	*P. psychrophila*	—	Spain
4	*P. quercina*	—	Spain
6	*P. taxon Pgchlamydo* *P. gonapodyides* *P. megasperma* *P. rosacearum*	P-126 — MKDF-7 MKDF-148	Kent Spain Macedonia Macedonia
7	*P. cinnamomi* *P. cambivora*	SCRP 127 P-075	Dundee Unknown
8	*P. cryptogea* *P. ramorum*	E2 P-018	Aberdeen Kent

2.4. Co-Cultivation of A. migulanus and Plant Pathogens

A. migulanus Nagano and NCTC 7096 were tested for activity against 13 species of *Phytophthora* (Table 1). The method was adapted and modified from Alenezi et al. [17]. Briefly, 90-mm-diameter Petri dishes of PDA were divided by drawing a line 20 mm from the edge of the dish. Five replicate cultures were used per *Phytophthora* species for each bacterial strain. An aliquot (10 µL) of overnight *A. migulanus* culture was inoculated linearly on the agar immediately over the line, and we left the bacterial line to dry for 3 min under sterilized condition. The line drawn was on the bottom of the Petri dish, and the density (colony-forming units (CFU)) of bacteria was 10^8 CFU mL^{-1}. Cultures were incubated at 37 °C for 48 h before subculturing a *Phytophthora* species to the dish by transferring a 6-mm-diameter disc to the opposite edge of the PDA. All cultures were incubated at 25 °C, except those of *P. psychrophila*, which were incubated at 20 °C. Graduated digital calipers were used to measure the distance in mm between bacterial strains and mycelial growth (inhibition zone) at time intervals of 2 days.

2.5. Effects of A. migulanus Treatment on Phytophthora Disease Severity on Apple Fruits

The ability of *A. migulanus* strains (Nagano, NCTC7096, and E1) to inhibit the development of disease was tested using a modified pathogen-baiting technique based on apples [28]. Granny Smith apples were washed with 70% ethanol followed by distilled water. The core of the tissue was then removed approximately 16 mm in depth aseptically with a sterilized 18-mm cork borer. The wound was inoculated with 500 µL of washed bacterial cell suspension (Nagano, NCTC, and E1), and the excised core was replaced. Apples were incubated at room temperature overnight before the inoculation with 6-mm-diameter mycelial discs of the *Phytophthora* species previously grown on 5% V8 medium [29]. For each test of the *Phytophthora* species, 10 replicate apples were inoculated with the pathogens. Negative controls were treated with 500 µL of distilled water. Treated apples were incubated in the dark at 25 °C, and the amount of rot that occurred was assessed by measuring the diameter of the externally visible lesions using graduated digital calipers at 48-h intervals. After 6 days, whole apples were weighed before excising and weighing the rotten tissues to calculate the proportion of rot.

2.6. Preparation of Gramicidin S Standard Curve

Gramicidin hydrochloride was obtained from Sigma-Aldrich (Sigma-Aldrich, Buchs, Switzerland). A 1-mM GS hydrochloride solution was obtained by dissolving commercial GS in 10 mL of sterile distilled water and filtering it through a 0.22-µm membrane filter

(Millipore, Scotland, UK). The dilutions of 10, 30, 50, 100, 300, 500, and 700 μM were then prepared: 1 mL of each dilution was submitted to LC-MS analysis and the peaks' areas were determined by the total ion current. At least five replicates of each solution were performed, and the calibration curve was prepared.

2.7. Extraction of Gramicidin S from A. migulanus Cultures

Overnight, *A. migulanus* Nagano or NCTC 7096 cultures were transferred to 250-mL Erlenmeyer flasks filled with 100 mL of TSB. Flasks were then incubated at 37 °C, with shaking at 180 rpm. After overnight incubation, 1 mL of the resulting cultures was centrifuged (3000× *g* for 10 min) and the supernatant was removed. The pellet was then dissolved in 1 mL of ethanol and incubated in a water bath at 70 °C for 15 min. After cooling to room temperature, the tubes were centrifuged in the same conditions as above, the ethanol extract was transferred to a new tube, and the ethanol was evaporated to dryness at room temperature by rotary evaporation at 45 °C. The residue was dissolved in 1 mL of ethanol and subjected to LC-MS analysis.

2.8. LC-MS Analysis

A reversed-phase column (Pursuit XRs ULTRA 2.8, C18, 100 mm × 2 mm, Agilent Technologies, Cheadle, UK) was used to perform HPLC analysis with the following conditions: a sample injection volume of 20 μL and column temperature of 30 °C. The mobile phases selected were 0.1% formic acid in water (A) and 0.1% formic acid in MeOH (B). For separation, a gradient program was used at a flow rate of 1 mL/min. The elution conditions employed a gradient of 100% solvent A/0% solvent B (25 min) to 0% solvent A/100% solvent B (5 min) over 20 min. MS was operated in the positive ion mode in a mass range of *m/z* 100–2000. A Thermo Instruments ESI-MS system (LTQ XL/LTQ Orbitrap Discovery, Waltham, MA, USA) connected to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump) was used to generate high-resolution mass spectral data.

2.9. Statistical Analysis

Graphs were plotted using Sigma plot 12.0. The *t*-tests and analysis of variance (ANOVA, Tukey test) were used to compare the inhibition ability of two bacterial treatments against *Phytophthora* spp. *in vitro* and *in planta*. All statistical tests were carried out using Minitab 17.

3. Results

3.1. Growth Characteristics of A. migulanus Nagano, NCTC 7096, and E1 Mutant

A. migulanus Nagano, NCTC 7096, and E1 mutant strains grew vigorously in TSB broth. The exponential phase of growth was observed after 2 h of subculturing. After 48 h of growth, the maximum number of CFU was reached with a density of 10^8 mL^{-1}. Cultures of *A. migulanus* Nagano, NCTC 7096, and E1 mutant entered approximately 8 h after subculturing using both CFU counts and OD measurements. It was obvious that there was no significant difference among the growth of the Nagano, NCTC 7096, and E1 mutant strains ($p > 0.05$) (Figure 1A,B).

3.2. Comparison of Biosurfactant Activity of A. migulanus Nagano, NCTC 7096, and E1 Mutant Strains Using Surface Tension Method

Surface tension of the culture fluids of the *A. migulanus* Nagano strain declined severely after 5 h of subculturing (Figure 1C). The stabilizing surface tension value was approximately 36 mNm^{-1} after 12–24 h of growth. The culture fluids of the NCTC 7096 strain showed a surface tension that began to decline after 12 h of subculturing with a final reduction to approximately 44 mNm^{-1}, whereas the surface tension of the culture fluids of the *A. migulanus* Nagano E1 mutant did not show any decline over all the culture period, with a constant value of 54 mNm^{-1}.

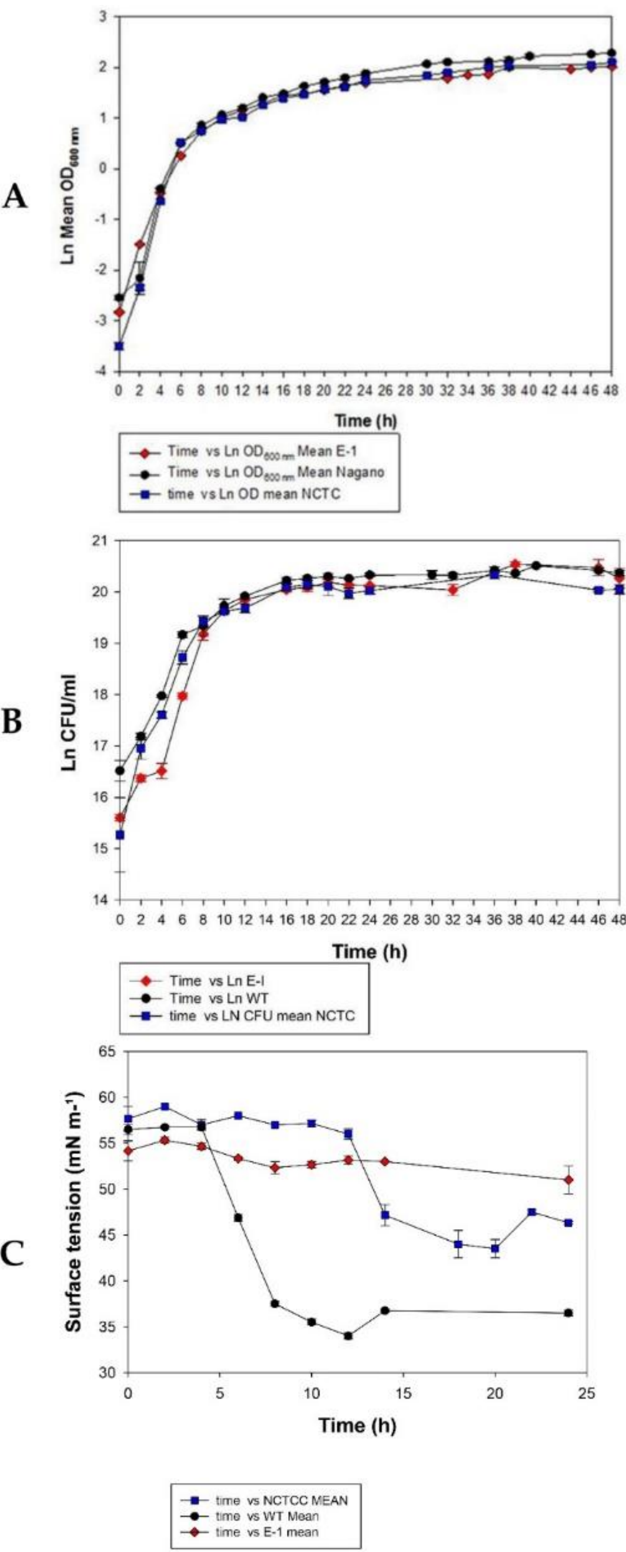


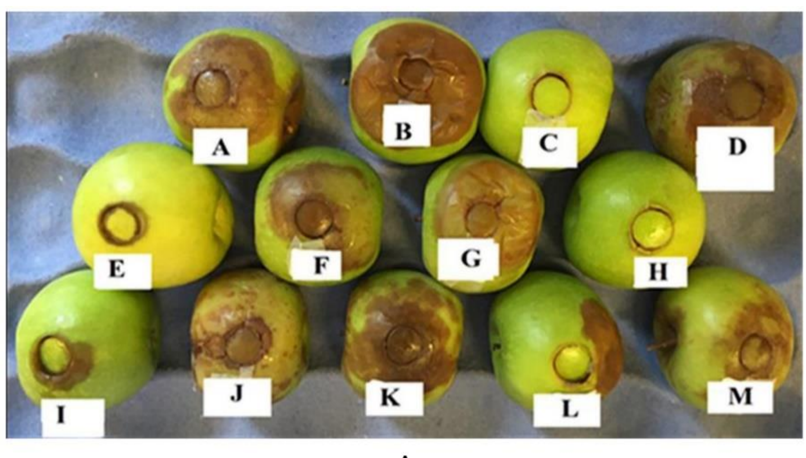
Figure 1. The comparison of growth rates among the three strains of *A. migulanus.* (**A**) Optical density (OD_{600nm}) measurements for (•) *A. migulanus* Nagano, (■) NCTC 7096, and (♦) E1. (**B**) Colony forming units (CFU) per mL for (•) *A. migulanus* Nagano, (■) NCTC 7096, and (♦) E1. Vertical bars represent standard errors of the means of three independent replicates. (**C**) *Aneurinibacillus migulanus* biosurfactant effects on surface tension of culture fluids and time course of changes in surface tension of (•) *A. migulanus* Nagano, (■) NCTC 7096, and (♦) E1 in culture fluids. Vertical bars represent standard errors of the means (N = 3).

3.3. Gramicidin Production by *A. migulanus* Strains

A. migulanus Nagano and NCTC 7096 both were proven as effective in producing GS in contrast to the *A. migulanus* Nagano E1 mutant (Supplementary Table S1).

3.4. Effect of *A. migulanus* Nagano and NCTC 7096 Strains on Apple Infection of *Phytophthora* Species

All *Phytophthora* species were more significantly inhibited by *A. migulanus* Nagano compared to NCTC 7096 ($p < 0.05$; Figure 2). Species belonging to clade 6 of *Phytophthora* were the most sensitive *Phytophthora* species to *A. migulanus* Nagano, whereas clade 2 species were the least sensitive (Figure 2). The highest inhibition caused by *A. migulanus* Nagano was on *P. psychrophila* at 20 °C, whereas the least inhibition was against *P. citrophthora* P-139 (Figure 2). Mutant E1 failed to inhibit the growth of all tested *Phytophthora* species (Figure 2).



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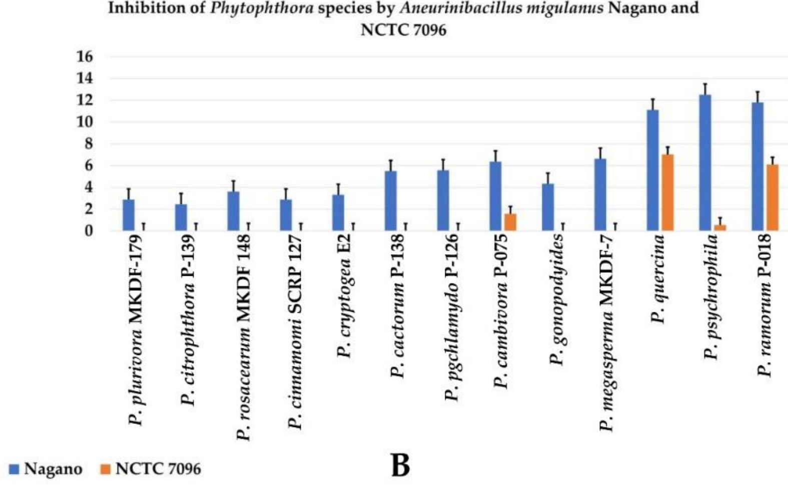

Figure 2. (**A**) In vivo pathogenicity assay of *Phytophthora* species on apple fruits; (A) *P. cryptogea* E2, (B) *P. rosacearum* MKDF-148, (C) *P. quercina*, (D) *P. plurivora* MKDF-179, (E) *P. cinnamomi* SCRP 127, (F) *P. cambivora* P-075, (G) *P. taxon Pgchlamydo* P-126, (H) *P. psychrophila*, (I) *P. megasperma* MKDF-7, (J) *P. citrophthora* P-139, (K) *P. cactorum* P-138, (L) *P. gonapodyides*, (M) *P. ramorum* P-018 (7 days after inoculation at 20 °C). (**B**) Inhibition of *Phytophthora* species by *A. migulanus* in screening assay. Vertical bars represent standard errors of the means (N = 5).

3.5. Effect of *A. migulanus* Nagano, NCTC 7096, and E1 Mutant Strains on Apple Infection of *Phytophthora Rosacearum* and *Phytophthora Cryptogea* Species

A. migulanus Nagano significantly reduced the external visible rot lesion sizes occurring in apples 6 days after inoculation with *P. rosacearum* MKDF-148 or *P. cryptogea* E2

(p = 0.001 and 0.01, respectively) compared with both apples being treated with *A. migulanus* NCTC 7096, positive controls, and mutant E1 (Figures 2 and 3). In contrast, there was no significant difference in the size of the rot lesions in the NCTC 7096, positive control, and mutant E1 as a negative control (*Phytophthora* inoculated apples) with both *Phytophthora* spp. (Figure 2).

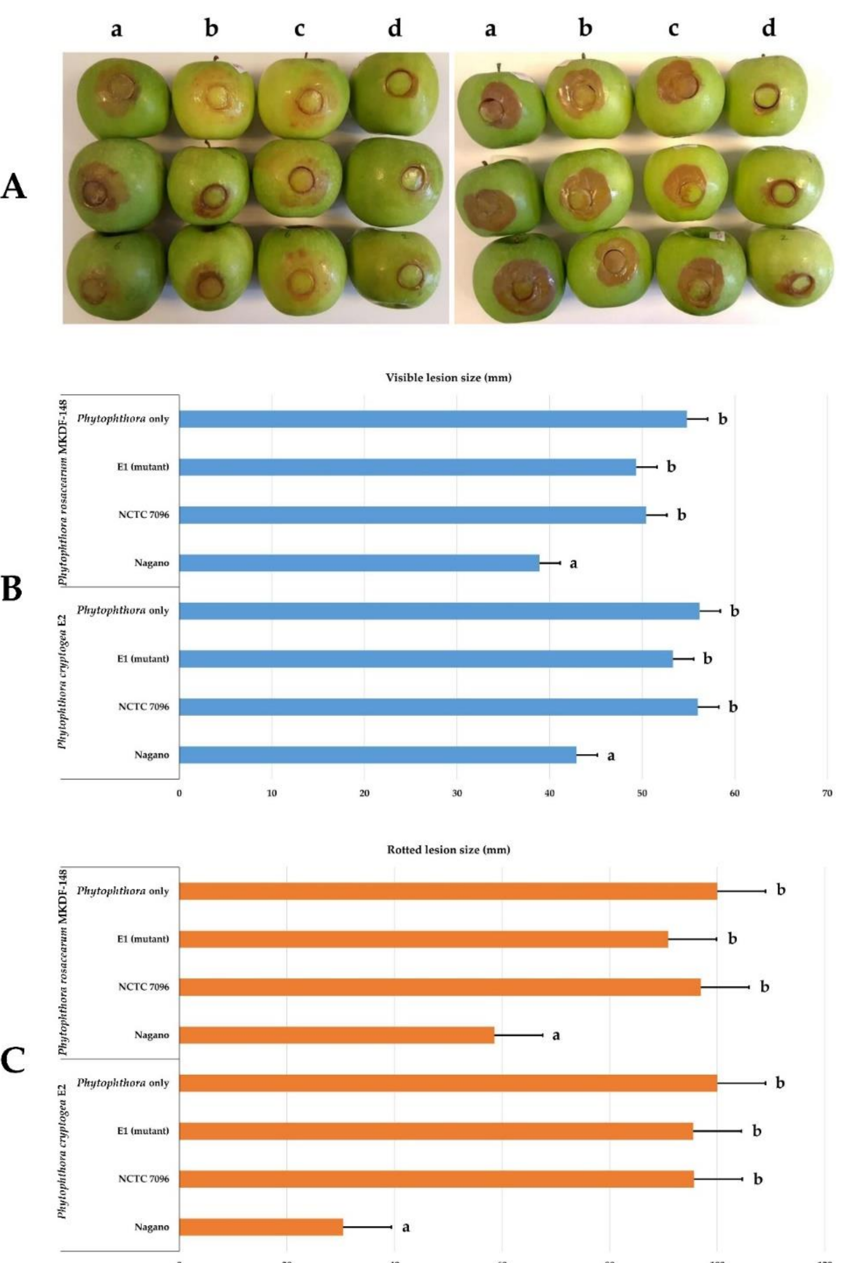

Figure 3. (**A**) Granny Smith apples inoculated with *P. cryptogea* (left side) and *P. rosacearum* (right side); (a) inoculated with *P. cryptogea* or *P. rosacearum* (positive control), (b) pre-treated with *A. migulanus* E1, (c) pre-treated with *A. migulanus* NCTC 7096, (d) pre-treated with *A. migulanus* Nagano (6 days after inoculation). (**B**) Effects of *A. migulanus Nagano*, NCTC 7096, and mutant E1 on lesion sizes in apples by *Phytophthora cryptogea* E2 and *P. rosacearum* MKDF-148 (6 days after inoculation). (**C**) Effects of *A. migulanus Nagano*, NCTC 7096, and mutant E1 on the amount of rot caused *by Phytophthora cryptogea* E2 and *P. rosacearum* MKDF-148 (6 days after inoculation). Bars labeled with different letters represent significant differences among the treatments at $p < 0.05$ using the Tukey's HSD test. In each bar groups, bars labeled with the same letter are not significantly different from each other according to Tukey's HSD at $p < 0.05$.

A. migulanus Nagano significantly reduced the amount of rot occurring in apples 6 days after inoculation with *P. rosacearum* MKDF-148 or *P. cryptogea* E2 ($p = 0$) compared with both apples being treated with *A. migulanus* NCTC 7096 and positive controls (Figure 2). In contrast, there was no significant difference in the quantity of rot in the NCTC 7096, positive control, and mutant E1 in apples with both *Phytophthora* species (Figure 2).

4. Discussion

Neither the use of the genetic resistance of the host nor the use of environmentally damaging synthetic pesticides succeeded in providing sustainable agricultural and forestry practices [3,7,30]. Therefore, biological control is more and more considered as a sound alternative of current practices for the management of plant pathogens in agricultural and forest ecosystems [6,8,11,13,31].

Biocontrol agents can act and positively impact plant health through well-established mechanisms, namely, antibiosis [2,18,32,33], induction of systemic resistance [34,35], and competition for nutrients and niches [4,5,10,13,17,36]. However, it is more and more documented that biocontrol is a multifactorial and complex process that has been until now under-investigated. Therefore, studies targeting the precise and fine study of biocontrol mechanisms are warranted towards the development of sustainable agricultural practices based on the use of biocontrol agents.

A. migulanus was shown to be a potent biocontrol agent effective in inhibiting various plant pathogens in agricultural and forest ecosystems [37]. Detailed studies of *A. migulanus* suggests that the bacteria act through the biosynthesis of a potent cyclic peptide, GS [17,22]. GS was shown to have a direct effect on pathogen spore germination and viability [17]. Apart from direct antibiosis, Seddon et al. [38,39] suggested that GS biosurfactant activity increases the rate of evaporation from the plant surface, thereby reducing periods of surface wetness inhibiting indirectly pathogen spore germination and viability. Indirect proof of the importance of GS in the interaction between *A. migulanus* and plant pathogens and, therefore, its biocontrol potential arises from the whole genome sequencing of *A. migulanus* Nagano and NCTC 7096 [25,26]. Up to 8% of their genome was dedicated to the biosynthesis of an impressive array of antimicrobial compounds, notably GS [17]. The availability of the *A. migulanus* Nagano GS mutant strain (E1 mutant) prompted us to study, for the first time, the importance of GS in the biocontrol ability of *A. migulanus* Nagano. LC-MS analysis of GS production in all *A. migulanus* tested strains allowed us to conclude that *A. migulanus* Nagano and *NCTC 7096* both proved to be effective in producing GS in contrast to the *A. migulanus* Nagano E1 mutant, which failed to produce significant amounts of GS (Table 2, Supplementary Tables S2 and S3). It is worth noting that there was a clear correlation between GS production by the *A. migulanus* strains and biocontrol ability in a previous study by Alenezi et al. [17]. These results were confirmed in this study including the E1 mutant. Using a surface tension assay, we were able to prove that the surface tension of the culture fluids of the *A. migulanus* Nagano strain declined severely after 5 h of subculturing.

Table 2. Nagano gramicidins' mass spectrometric peptide fragmentation. For GS-1141, GS-1155, and GS-1169, where (■) represents the presence of each fragment, (■) represents the absence and (■) represents the actual mass.

Title	**Nterm_mod**	**aa1**	**aa2**	**aa3**	**aa4**	**aa5**	**aa6**	**aa7**	**aa8**	**aa9**	**aa10**	**adduct**
	Term	**L**	**F**	**P**	**V**	**K**	**L**	**F**	**P**	**V**	**K**	**$[M + H]^+$**
Mass	1.007825	113.0841	147.0684	97.05276	99.06841	128.095	113.0841	147.0684	97.05276	99.06841	128.09496	1169.745
b-series	1.007825	114.0919	261.1603	358.2131	457.2815	585.3764	698.4605	845.5289	942.5817	1041.65	1169.745	
Actual mass		-	261.1606	358.2188	457.2803	585.3751	698.4605	845.5264	942.5762	1041.6481		
y-series	1.007825	1169.745	1056.661	909.5926	812.5398	713.4714	585.3764	472.2924	325.224	228.1712	129.102785	
Actual mass		-	1056.6727	909.5892	812.5347	713.4695	585.3751	472.2917	325.2228	228.1687		
	Nterm_mod	aa1	aa2	aa3	aa4	Orn	aa6	aa7	aa8	aa9	aa10	adduct
	Term	L	F	P	V	K	L	F	P	V	K	$[M + H]^+$
Mass	1.007825	113.0841	147.0684	97.05276	99.06841	114.0793	113.0841	147.0684	97.05276	99.06841	128.09496	1155.729
b-series	1.007825	114.0919	261.1603	358.2131	457.2815	571.3608	684.4448	831.5132	928.566	1027.634	1155.7294	
Actual mass		-	261.1598	358.2122	457.2795	571.3612	684.4433	831.5117	928.5595	1027.6339		
y-series	1.007825	1155.729	1042.645	895.5769	798.5241	699.4557	585.3764	472.2924	325.224	228.1712	129.102785	
Actual mass			1042.6502	895.5762	798.5214	699.4553	585.3752	472.2917	325.2232	228.1713		
	Nterm_mod	aa1	aa2	aa3	aa4	Orn	aa6	aa7	aa8	aa9	Orn	adduct
	Term	L	F	P	V	K	L	F	P	V	K	$[M + H]^+$
Mass	1.007825	113.0841	147.0684	97.05276	99.06841	114.0793	113.0841	147.0684	97.05276	99.06841	113.93846	1141.573
b-series	1.007825	114.0919	261.1603	358.2131	457.2815	571.3608	684.4448	831.5132	928.566	1027.634	1141.5729	
Actual mass			261.1598	358.2108	457.2784	571.3607	684.4442	831.5147	928.5653	1027.6261		
y-series	1.007825	1141.714	1028.63	881.5613	784.5085	685.4401	571.3608	458.2767	311.2083	214.1555	115.087135	
Actual mass			1028.6317	881.5618	784.5082	685.4402	571.3607	458.2810	311.2080	214.1554		

The stabilizing surface tension value was approximately 36 mNm^{-1} after 12–24 h of growth. The culture fluids of the NCTC 7096 strain showed a surface tension that began to decline after 12 h of subculturing, with a final reduction to approximately 44 mNm^{-1}, whereas the surface tension of the culture fluids of the *A. migulanus* Nagano E1 mutant did not show any decline over all the culture periods, with a constant value of 54 mNm^{-1}. This result is in agreement with the report of Alenezi et al. [17], where, additionally, the *A. migulanus* Nagano dried significantly faster on the tomato leaf surface than did the *A. migulanus* NCTC 7096 and E1 mutant [17]. This result clearly documented that the E1 mutant was impaired in the production of GS and its associated biosurfactant activity. Additional confirmation of this result was obtained using blood Agar and oil spreading methods applied to the *A. migulanus* Nagano, NCTC 7096, and E1 mutant strains. Although both strains, *A. migulanus* Nagano and NCTC 7096, were able to spread oil on the surface of water, the E1 mutant was not able do so, suggesting that the E1 mutant lost totally its biosurfactant activity. It is worth noticing that the diameter of the oil spread was greater for *A. migulanus* Nagano than for NCTC 7096 (p = 0.00; two-sample t-test), as reported by Alenezi et al. [17].

Using a set of 13 Phytophthora species, namely, *P. cactorum*, *P. plurivora*, *P. citrophthora*, *P. psychrophila*, *P. quercina*, *P. taxon Pgchlamydo*, *P. gonapodyides*, *P. megasperma*, *P. rosacearum*, *P. cinnamomi*, *P. cambivora*, *P. cryptogea,* and *P. ramorum,* we unambiguously documented that all Phytophthora species were more significantly inhibited by *A. migulanus* Nagano compared to NCTC 7096 ($p < 0.05$). This result is in agreement with further reports [17,22], suggesting the strain level biocontrol abilities in *A. migulanus*. Interestingly, species belonging to clade 6 of Phytophthora were the most sensitive Phytophthora species to *A. migulanus* Nagano, whereas clade 2 species were the least sensitive (data not shown). We speculated that clade-specific determinants are targeted by *A. migulanus*. However, this needs further targeted studies to determine the reasons of clade specificity of Phytophthora to *A. migulanus*. Our results also clearly document that the highest inhibition caused by *A. migulanus* Nagano was on *P. psychrophila* at 20 °C, whereas the least inhibition was against *P. citrophthora* P-139. Based on these results, two phytophthora species highly sensitive to *A. migulanus* (*P. rosacearum* and *P. cryptogea*) were selected for the rest of the study. These two species have also the advantage of being the most aggressive on apple fruits, displaying clear infection halos. It is worth noting that the *A. migulanus* Nagano mutant E1 failed to inhibit the growth of all the tested Phytophthora species (Figure 3). Further experiments using the combination of the two Phytophthora species with *A. migulanus* Nagano, NCTC 7096, and E1 mutants clearly indicated that the *A. migulanus* Nagano E1 mutant impaired in the biosynthesis of GS was unable to inhibit the growth of both Phytophthora species. *A. migulanus* Nagano significantly reduced the external visible rot lesion sizes occurring in apples 6 days after inoculation with *P. rosacearum* MKDF-148 or *P. cryptogea* E2 (p = 0.001 and 0.01, respectively) compared with both apples being treated with *A. migulanus* NCTC 7096, positive controls, and mutant E1 (Figures 1 and 2).

5. Conclusions

The present work revealed a negative correlation between GS production in the culture fluids of *A. migulanus* and its biocontrol ability. This discovery provides a direct proof of the importance of GS for the biocontrol ability of *A. migulanus*. It also paves the way towards the development of effective biocontrol agents either in a homologous or heterologous way by overexpressing the GS biosynthetic cluster. Ongoing results that will be published in a future manuscript confirm this speculation and provide a model for further similar investigations in closely related species and genera.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f13040535/s1. Table S1: NCTC 7096 gramicidins' mass spectrometric peptide fragmentation. For GS-1141, GS-1155, and GS-1169, where (■) represents the presence of each fragment and (■) represents the absence and (■) represents the actual mass. Table S2: E1 mutant (non-gramicidin's producer) gramicidins' mass spectrometric peptide fragmentation. For GS-1141, GS-1155, and GS-1169, where (■) represents the presence of each fragment, (■) represents the absence and (■) represents the actual mass.

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