Intracellular and extracellular expression of *Bacillus thuringiensis* crystal protein Cry5B in *Lactococcus lactis* for use as an anthelminthic

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Running title: Expression of Bt crystal protein Cry5B in *Lactococcus*

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Abstract

Bacillus thuringiensis crystal (Cry) proteins, Cry5B (140 kDa) and a truncated version of the protein (tCry5B, 79 kDa) are lethal to nematodes. Genes encoding the two proteins were separately cloned into a high copy vector with a strong constitutive promoter, pTRK593, in *Lactococcus lactis* for potential oral delivery against parasitic nematode infections. Western blots using a Cry5B specific antibody revealed that constitutively expressed Cry5B and tCry5B were present in both cells and supernatants. To increase production, *cry5B* was cloned into high copy number plasmid pMSP3535H3, encoding a nisin-inducible promoter. Immunoblotting revealed that 3 hours after nisin induction, intracellular Cry5B was strongly induced at 200 ng/ml nisin, without adversely affecting cell viability or cell membrane integrity. Both Cry5B genes were also cloned into plasmid pTRK1061, encoding a promoter and transcriptional activator that

invokes low expression of prophage holin and lysin genes in *Lactococcus* lysogens, resulting in a leaky phenotype. Cry5B and tCry5B were actively expressed in the lysogenic strain *L. lactis* KP1, and released into cell supernatants without affecting culture growth. Lactate dehydrogenase (LDH) assays indicated that Cry5B, but not LDH, leaked from the bacteria. Lastly, using intracellular lysates from *L. lactis* cultures expressing both Cry5B and tCry5B, in vivo challenges of *Caenorhabditis elegans* (*C. elegans*) demonstrated that the Cry proteins were biologically active. Taken together, these results indicate that active Cry5B proteins can be expressed intracellularly and released extracellularly in *L. lactis*, showing potential for future use as an anthelminthic that could be delivered orally in a food-grade microbe.

Introduction

Intestinal roundworm (nematode) parasites, including hookworms, whipworms and *Ascaris*, infect over a billion people worldwide, and negatively affect growth, nutrition, cognition and pregnancy (1). The World Health Organization has approved four anthelmintic drugs to treat roundworm infections, two benzimidazoles and two nicotinic acetylcholine receptor agonists (2). Resistance to these drugs is already widespread, leading to an urgent need for the development of new anthelmintics (2), and efforts are underway to develop *Bacillus thuringiensis* (Bt) crystal (Cry) proteins to treat intestinal nematode infections in humans. *B. thuringiensis* is a gram-positive, spore forming bacterium that produces para-sporal crystalline protein inclusions known as Cry proteins (3). The Cry proteins are pore-forming proteins that bind to receptors on the intestines of invertebrates, causing impairment or death. A number of Cry proteins have been found to provide effective treatments against nematodes, and more specifically against parasitic helminths, in vivo (4, 5). Cry proteins have been shown to produce lethargy, anorexia, pale

coloration, brood size reduction, developmental arrest and/or death of roundworms. Cry proteins are proven to be safe for human consumption after over 50 years use as crop insecticides, in aerial spraying, and incorporated into transgenic food crops, including organic crops (3).

One Bt Cry protein, Cry5B, has been shown to significantly reduce parasite burdens of 1) mice infected with the natural intestinal parasite *Heligmosomoides polygyrus bakeri;* 2) hamsters infected with the zoonotic hookworm parasite *Ancylostoma ceylanicum*; and 3) pigs infected with *Ascaris* (6, 7). Cry5B is a three-domain Cry protein similar in structure to the Cry1 family used in transgenic crops (3, 8). The genetic receptors for Cry5B in nonparasitic roundworm *Caenorhabditis elegans* are carbohydrate structures present on lipids found on the roundworm intestinal surface, structures which are absent in vertebrates (8). After binding to the receptors, protein monomers are proteolytically processed, oligomerize, and form pores in the plasma membrane of the intestine, causing death or severe damage to the intestinal surface of the nematode (8). A truncated version of Cry5B was cloned for expression in plant tissues and found to be highly toxic to *C. elegans* (9). The C- terminal truncated version retains the first 2094 nucleotides of *cry5B*, and the five conserved amino acid motif, DRIEF, known as block 5 which occurs at the end of the active toxin domain of the Cry proteins (9, 10).

The food grade bacterium *Lactococcus lactis* has been used to express a variety of vaccines and biotherapeutics (11-13). *L. lactis* does not colonize mammalian digestive tracts (14). Colonization is not a desirable feature for a bacterial protein delivery system to the GIT. Ideally, the live delivery bacteria would be administered, survive for some time, and then would be washed through and out of the GIT. *L. lactis* is known to be acid and bile sensitive and is thus vulnerable in the mammalian stomach and small intestine (15). However, this susceptibility is

strain dependent and can be altered when *L. lactis* is protected with milk or food, either in vitro or in vivo (15-17). *L. lactis* can be incorporated into a food matrix, into a fermented milk product, or freeze-dried and encapsulated (18, 19). In the GIT, Cry5B containing *L. lactis* would release the protein upon lysis (15, 16). Alternatively, Cry5B could be exported from surviving *L. lactis* cells into the GIT, or killed *L. lactis* cells could be administered as an abiotic (20). Interestingly, viable *L. lactis* is metabolically active in each compartment of the rat GIT, while dead cells are rapidly lysed (16). Recent studies have shown that even dead bacteria when administered as probiotics can have a significant effect on the host, particularly on the immune system (20, 21). Thus, either live or dead, *L. lactis* cells could function as a safe delivery vehicle for Cry5B, which would eventually be released directly into the intestinal environment of the helminth population.

Here we test the hypothesis that such a food grade bacterium could be engineered to express and release full length Cry5B and its truncated form, (tCry5B), potentially to target nematode parasites in the gastrointestinal tract (GIT). This study presents two systems in *Lactococcus lactis* developed for the successful intracellular expression and externalization of full length and truncated Cry5B.

Methods

Bacterial strains and culture conditions. Strains and plasmids used in this work are shown in Table 1. *L. lactis* MG1363 and KP1 were propagated aerobically without shaking at 30° C in Difco M17 medium (Becton, Dickinson and Company, Sparks, MD) supplemented with 5% glucose (M17G). *E. coli* DH5α (Life Technologies, Grand Island, NY) and XLI-Blue (Agilent

Technologies, Inc., Santa Clara, CA), were propagated with shaking in Luria-Bertani broth (LB) or on Bacto Brain Heart Infusion (BHI) agar plates at 37° C (Becton Dickinson). Cultures were stored at -20°C in the appropriate medium supplemented with 10% glycerol. As necessary, growth media were supplemented with 1.5 or 150 ug/ml erythromycin (Em) for *L. lactis* or *E. coli*, respectively. Growth curves were produced either in test tubes using a Bausch and Lomb Spectronic 20 or using a FLUOstar OPTIMA plate reader (BMG LABTECH, Inc., Durham, NC).

Plasmid construction. Primers for PCR cloning were obtained from Integrated DNA Technologies (Coralville, IA). PCR was accomplished using the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Inc.). Restriction enzymes were obtained from Roche Applied Science (Indianapolis, IN). DNA fragments were gel purified using the QIAquick Gel Extraction Kit, and PCR reactions were purified using the QIAquick PCR Purification Kit (QIAGEN Sciences, Valencia, CA). DNA ligations were carried out with the Fast-Link DNA Ligation Kit (EPICENTRE Biotechnologies, Madison, WI). The ligation mixes were transformed into *E. coli* XL1-Blue or DH5α using the Z-Competent *E. coli* Transformation Buffer Set (Zymo Research, Orange, CA). Transformation isolates were selected on BHI agar plates with 150 ug/ml Em, and propagated in LB with 125 ug/ml Em. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit. Cloning isolates were confirmed by sequencing at Eton Biosciences (Research Triangle Park, NC) or Davis Sequencing (Davis, CA), directly from plasmid DNA or from PCR products obtained using Choice Taq Blue DNA polymerase (Denville Scientific, Metuchen, NJ). Plasmids were visualized *in silico*, and DNA sequences were aligned using the Clone Manager 9 Professional Edition (Scientific and Educational Software, Cary, NC) and the Geneious Pro software packages (Biomatters Ltd., Auckland, NZ).

111 Confirmed plasmids were electroporated into *L. lactis* as described previously (22). Plasmids
112 were isolated from *L. lactis* transformants using the QIAprep Spin Miniprep Kit, following the
113 manufacturer's instructions, but with an added 15 min incubation in buffer P1 at 37°C with 5-6
114 mg lysozyme (Sigma-Aldrich Co, St. Louis, MO) prior to the addition of buffer P2. The *L. lactis*
115 clones were confirmed by sequencing PCR products amplified from the plasmid DNA using the
116 M13 F and M13 R primers.

117 The full length (3792 bp) and truncated (2126 kb) versions of the *cry5B* gene were initially
118 cloned into pTRK593 downstream of the constitutive P6 promoter (23). PCR products were
119 amplified from pQE9-Cry5B with primers encoding an upstream SalI site, ribosome binding site,
120 and start codon (forward primer Cry5B-RBS-SalI
121 GATC*GTCGAC*AAGGAGAACGTATATGGCAACAATTAATGAGTTGTATC); and with
122 downstream PstI sites (reverse primer for full-length Cry5B: pQE9-R-PstI
123 GATC*CTGCAG*TATCCAAGCTCAGCTA, reverse primer for truncated Cry5B: pQE9-R-
124 tCry5B: GATC*CTGCAG*ATCAGTCTATTGGATT). The truncated version of Cry5B ends 2 aa
125 residues after the box 5 feature of Cry5B, and is followed by a stop codon (9). The Cry5B PCR
126 products were cloned into Stratagene plasmid pSC-B via the StrataClone Blunt PCR Cloning Kit
127 following the manufacturer's instructions (Agilent Technologies, Inc, Santa Clara, CA), and
128 from there cloned into pTRK593 (23). The *cry5B* insert DNA was removed from pSC-B with
129 PstI/SalI/BglI. BglI was used to digest the pSC-B vector into smaller pieces since it is almost the
130 same size as the *cry5B* fragment. The truncated cry5B insert was removed with PstI/SalI. Each
131 insert was ligated into PstI/SalI digested pTRK593 DNA.

Cloning for Cry5B expression by leaky *Lactococcus* was accomplished using vector pTRK1061. The vector was constructed from pTRK617 (24) using an XhoI/SalI double digest and religation, removing the TTFC gene. The following primers were used to amplify cry5B from pTRK1040: forward, Cry5B RBS PstI : GATC*CTGCAG*AAGGAGAACGTATATGGCAACAATTAATGAGTTGTATC and reverse, Cry5B XhoI R: GATC*CTCGAG*TATCCAAGCTCAGCTAATTAAG. The reverse primer for truncated Cry5B was tCry5B XhoI R: GATCCTCGAGATCAGTCTATTGGATTTTTGGAAC. The Cry5B fragments and vector pTRK1061 were digested with PstI and XhoI and ligated.

For nisin induced expression of Cry5B using vector pMSP3535H3, full length and truncated cry5B were PCR amplified from pTRK1040 with the following primers: cry5B SphI forward: GATC*GCATGC*GTGAGGAGAACGTATATGGCAACAATTAATGAGTTG and cry5B BamHI reverse: GATC*GGATCC*GCAGTATCCAAGCTCAGCTAATTAAG. Truncated Cry5B was amplified with truncated cry5B BamHI reverse: GATC*GGATCC*GCAGTATCCAAGCTCAGCTAATTAAG. The PCR products were cloned using the StrataClone Blunt PCR Cloning Kit into vector pSC-B, and from there to pMSP3535H3 using the SphI and BamHl restriction sites encoded in the PCR primers.

Immunoblotting. *L. lactis* cultures were propagated 16 h and then transferred from overnight cultures and propagated in 10 ml M17G with Em (1.5 ug/ml) to OD_{600} 0.5-0.6 (log phase), followed by centrifugation to pellet cells. Supernatants and pellets were frozen separately at -80° C for storage. Cell supernatants were filtered through 30,000 MW cut-off Amicon Ultra centrifugal filters following the manufacturer's instructions (Millipore Ireland Ltd., Tullagreen, Ireland). The filters were then washed with 4 ml of PBS, pH 7.4 (Invitrogen, Grand Island, NY),

and the concentrated proteins eluted with 50 ul of PBS. Cell pellets were resuspended in 1 ml PBS and were homogenized with the Mini BeadBeater 8 using 0.1 mm glass beads in 2 ml screw cap tubes for 3 one minute intervals interspersed with 1 min on ice to produce cell lysates (Biospec Products, Bartlesville, OK). Protein concentration in all samples was determined using the Bradford Reagent (Sigma-Aldrich Co., St. Louis, MO), and approximately 7-10 ug protein per lane was electrophoresed on Mini-Protein 7.5% or 4.5-10% TGX gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes using the Bio-Rad Trans-Blot Turbo Transfer system following manufacturer's instructions. The membranes were hybridized with Cry5B primary antibodies (Thermo Fisher Scientific, Waltham, MA) and with Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (Bio-Rad) and developed with Pierce ECL Western Blotting Substrate (Fisher Scientific, Pittsburg, PA) or Clarity Western ECL Substrate (Bio-Rad) following the manufacturer's instructions.

Nisin induction. Stock solutions of 1 mg/ml nisin in 0.02 N HCl were prepared from a preparation of 2.5% nisin obtained from Sigma-Aldridge Corp. (N5764, St. Louis, MO). Ten ml cultures were grown to OD_{600} 0.3 - 0.4 in M17G with Em after inoculation from 18 hr cultures. Nisin was added at various dilutions to the cultures, and incubation continued for 3 hr before harvesting cells and supernatants for comparison of protein expression by Western Blotting. For growth curve experiments, control strains were grown to OD_{600} 0.4 and then incubated for an additional 3 hr without the addition of nisin. (final OD_{600} was 1.0-1.1).

Lactate dehydrogenase (LDH) activity assays. LDH activity was detected indirectly by measuring the oxidation of NADH in the presence of pyruvate and fructose 1,6-diphosphate (FDP) at 340 nm (25, 26). The protocol was modified for use with a well mode assay in a

FLUOstar OPTIMA plate reader. Supernatant samples were removed from centrifuged 1 ml log phase cultures (OD_{600} 0.5) and transferred to a separate tube. A reagent mix of Tris, pH 6.8 (0.05 M), sodium pyruvate (0.1 M), and FDP (30 mM, all chemicals from Sigma-Aldrich) was aliquoted at 223 ul per well into the wells of a 96 well plate. Samples (20 ul) of cell supernatants were added to the wells in triplicate. NADH (25 ul of 1.5 uM in Tris buffer, pH 8.5) was separately injected into each well, followed by 2 s of shaking and five consecutive OD_{340} readings spaced 30 sec apart. End points of the reactions were observed, and LDH activity was calculated from the slope of the kinetic curve for each well.

Live Dead assays. To assay cell permeability, the LIVE/DEAD *Bac*Light Bacterial Viability Kit (kit L7012, Life Technologies, Grand Island, NY) was used according to the manufacturer's instructions. The kit uses two nucleic acid-binding stains to differentiate cells with intact membranes (live cells) from those with compromised membranes (dead cells). SYTO 9 is a green, cell-permeant dye that labels all bacteria regardless of membrane integrity. Propidium iodide (PI) is a red dye which labels cells with compromised or damaged cell membranes. A standard curve was prepared according to the manufacturer's instructions using standard ratios of untreated log phase cells and log phase cells treated with 70% isopropyl alcohol for 1 hr. For the assay, 6-10 ml of log phase or treated cells was centrifuged, resuspended in 4 ml of 0.85% NaCl, and adjusted to OD_{690} of 0.3. 100 ul aliquots of adjusted cells were added in triplicate to the wells of a black 96 well plate (NUNC 267342, Thermo Scientific, Waltham, MA) with 100 ul of an equal mixture of kit reagents A and B (3.34 mM STYO 9 dye, 480/500 excitation/emission; and 20 mM propidium iodide, 490/635 excitation/emission) each diluted 3:2000 v:v in H_2O. After mixing, the plates were held 15 min in the dark and then read at 490/530 and 490/600 excitation/emission settings on a FLUOstar OPTIMA plate reader. Ratios of SYTO 9 signal

199 divided by PI signal were determined, and the results used to calculate the percent of undamaged
200 cells based on the standard curve.

201 ***C. elegans* assays.** *C. elegans* strain *sek-1 (km4)* was used in a bio-activity assay (27). SEK-1 is a
202 MAPK kinase (MAPKK) that is immediately upstream of the *C. elegans* p38 MAPK, PMK-1,
203 and immediately downstream of the MAPKK kinase, NSY-1 (28, 29). *C. elegans sek-1 (km4)*
204 was maintained using standard techniques with *E. coli* strain OP50 as a food source (30). Assays
205 were performed at 25°C as previously described for developmental assays (31), except that in
206 place of S medium, 200 ul of *L. lactis* MG1363 cell pellet lysate was added to each well. Cell
207 lysates were prepared in PBS as described above. *E. coli* OP50 cells were propagated to OD_{600}
208 3.0, and 40 ul added per well with 10-15 *sek-1 (km4)* first life stage larvae and 0.2 ul 5 mg/ml
209 cholesterol (in ethanol) as the food source. Assays were performed in 48 well plates with two
210 wells per condition in each of three independent trials. Images of *C. elegans* were captured using
211 an Olympus BX-60 microscope at 48 hr of incubation. The images shown in Figure 7 are
212 representative of the results over the three trials.

213 ## Results

214 **Expression of Cry5B and tCry5B from pTRK593 for high level, constitutive expression.**
215 Vector pTRK593 (23), which is based on the theta replicating plasmid pTRKH2 (32), replicates
216 in *E. coli* and *Lactococcus* with a high copy number. The genes encoding Bt Cry proteins, Cry5B
217 and truncated tCry5B were cloned behind a strong, constitutive promoter, P6, positioned
218 upstream of a multiple cloning site in pTRK593. Plasmids pTRK1040 (pTRK593::cry5B) and

pTRK1041 (pTRK593::tcry5B) were first obtained in *E. coli* and then transformed into *L. lactis* strains MG1363 and KP1.

Immunoblots confirmed intracellular expression of the Bt Cry proteins in both *Lactococcus* backgrounds (Fig. 1A, constitutive). Full length Cry5B is partially degraded in the *Lactococcus* intracellular samples (Fig. 1A), but not in the extracellular samples (Fig. 1B). The mechanism of degradation is unknown, but commonly occurs when heterologous proteins are expressed intracellularly in *L. lactis* (33). Significant proportions of the Cry5B and tCry5B proteins were also found in the cell supernatants of the log phase cultures from both lactococcal expression hosts (Fig. 1B, constitutive). The results clearly showed the expression and release of Cry5B and truncated Cry5B in *Lactococcus lactis* MG1363 and KP1.

Cry5B expression through nisin induction on a high copy number plasmid. In attempts to increase expression in *Lactococcus*, the Cry5B and tCry5B inserts were cloned into plasmid pMSP3535H3 (34). Plasmid pMSP3535H3 is a derivative of the original nisin expression vector pMSP3535, with an improved promoter, a bi-directional terminator, and a replication region derived from pTRKH2. Also encoded is the *nisI* nisin resistance gene, which allows induction with higher levels of nisin than was possible in the original vector (32, 34, 35). The plasmids produced using this vector are pTRK1068 (pMSP3535H3::cry5B) and pTRK1069 (pMSP3535H3::tcry5B). These plasmids were transformed into *L. lactis* MG1363.

The effect of added nisin on the *L. lactis* MG1363 cultures containing pMSP3535H3-based plasmids was investigated. Growth curves were generated using MG1363, MG1363 plus vector (pMSP3535H3), and *L. lactis* subsp. *lactis* ATCC 11454 (a nisin producing strain which is

naturally nisin resistant) and nisin added at 0, 50, and 200 ng/ml (Fig. 2A). The results show that the parent MG1363 was significantly inhibited by high levels of nisin, whereas MG1363 encoding the nisin expression vector grew uninhibited in high levels of nisin, up to 200 ng/ml, as did the naturally resistant, nisin producing control strain, ATCC 11454. Cell membrane permeability of nisin induced cells was tested using the LIVE/DEAD BacLight assay. This assay uses two fluorescent dyes: the green SYTO 9 which stains all cells, and red propidium iodide (PI) which is only taken up into cells that are membrane compromised. The ratio of the fluorescence from the two dyes is used to calculate the percent "live" cells from a standard curve. Here we used the assay as an indication of increased membrane permeability, as reflected by a lower percentage of "live", or membrane intact, cells. The percentage of membrane intact cells was only slightly affected by the addition of nisin to the log phase cells (Fig. 2B).

Immunoblots were used to investigate the effect of various levels of nisin induction on Cry5B expression (Fig. 3). At 50, 100, and 200 ng/ml nisin induction, Cry5B demonstrated strong induction, however the Cry5B protein appears to be processed, reducing its apparent size from 140 kD to about 120 kD. This processing was evident when Cry5B is expressed from the P6 promoter in pTRK1040 in both MG1363 and KP1 (lanes 3 and 4), but is more prominent after nisin induction (lanes 6-14). Cell supernatants were also tested, however significant amounts of Cry5B were not detected (data not shown). Varying amounts of cell lysate proteins were loaded to SDS-PAGE gels (2.5, 5, and 10 ug/lane) and immunobloting used to determine the relative amounts of Cry5B produced by nisin induction in MG1363 (nisin induced, pTRK1068) compared with the base level produced in either in *L. lactis* MG1363 or KP1 harboring the constitutive expression plasmid pTRK1040 (Fig. 3). Comparing nisin induced expression in MG1363 (pTRK1068, Fig.3, lanes on the right showing 10 ug protein/lane) with constitutive

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expression from MG1363 (pTRK1040, Fig. 3, left lanes, constitutive), showed that a significantly higher amount of total Cry5B was induced with 200 ng/ml nisin, as compared to the base expression vector. However, comparable amounts of full-length, 140 kDa, Cry5B were expressed from both pTRK1040 and pTRK1068 (compare Fig. 3, lanes 3 and 14).

Externalized expression of Cry5B using the leaky *Lactococcus* system. For use as an anthelminthic, we sought *L. lactis* strains that produced Cry5B intracellularly and also strains capable of exporting or externalizing Cry5B. The leaky *Lactococcus* system (24) was investigated for possible externalization of the Cry5B protein. Previously, *L. lactis* KP1 (NCK203) was shown to externalize TTFC (tetanus toxin fragment C) when expressed from pTRK617 (24). Plasmid pTRK617 contains an 888-bp fragment of lytic lactococcal bacteriophage ϕ31, which encodes a late phage promoter (P_{15A10}) and Tac31A, a phage transcriptional activator. The leaky phenotype depends on the presence in the lactococcal host strain of the plasmid encoded activator as well as a resident prophage which encodes a phage promoter homologous to P_{15A10}. Low level activation of the resident prophage holin and lysin cassette *in trans* from the plasmid produces the leaky phenotype (24). Leaky vector pTRK1061 was constructed through deletion of the TTFC gene in pTRK617. Plasmids pTRK1062 (pTRK1061::Cry5B) and pTRK1063 (pTRK1061::tCry5B) were produced and were transformed into *L. lactis* KP1, which contains at least two prophages, and was used as the plasmid host strain to leak TTFC in previous work (24).

Immunoblotting revealed that virtually all of the Cry5B and tCry5B produced in log phase KP1 with the leaky system appeared in the cell supernatant (Fig. 1B. leaky, pTRK1061 based), with little to none remaining in the cell pellet lysates (Fig. 1A leaky, pTRK1061 based). However, the

amount of Cry5B or tCry5B in the supernatants is not markedly greater than observed in the supernatants where Cry5B and tCry5B were expressed from the stronger P6 promoter of pTRK593. Similar to the previous experiments with TTFC externalization (24), cell growth was not affected (Fig. 4).

Cell membrane permeability assays using the LIVE/DEAD BacLight kit, were conducted to determine whether increased cell permeability contributed to the externalization of Cry5B in the leaky cells (Fig. 5). Increased permeability of leaky *L. lactis* KP1 log phase cells compared to non-leaky cells or to MG1363 cells was not detected (Fig. 5A). This result agrees with the previous study, where no increase in cell permeability was detected using propidium iodide with log phase cells (24). However, when overnight cells were tested (Fig. 5B), distinct differences were observed. Overnight *L. lactis* MG1363 cells were less permeable than in log phase, whereas KP1 cells were unchanged. For the overnight samples, KP1 was distinctly more permeable than MG1363, and KP1 encoding the leaky vector or leaky vector with Cry5B were significantly more permeable than KP1 without the leaky plasmids. These results might be expected for stationary phase cells exhibiting a holin/lysin induced leaky phenotype.

Lactate dehydrogenase (LDH, 35 kDa) assays were used to investigate whether or not Cry5B was released into the cell lysate through the normal process of cell lysis in a bacterial population. Normally, LDH is an intracellular enzyme, and not found in cell supernatants unless cell lysis occurs (36). LDH experiments repeated three times with log phase samples of the leaky strains and controls were used to compare LDH activity in the cell supernatants among the leaky and non-leaky strains (Fig. 6). Extracellular LDH activity was negligible in all the supernatant samples. These data indicate that cell lysis was not occurring in the log phase cells, and lysis

was not responsible for the external release of the Cry proteins, but likely occurred as a result of the phage activator Tac31A and its effect on the late holin and lysin genes of the resident KP1 prophage.

Biological Activity of Cry proteins

To assess the biological activity of Cry5B expressed in *L. lactis*, bioactivity assays of cell free lysates of *L. lactis* MG1363 producing Cry5B and tCry5B were performed using a laboratory roundworm model, *C. elegans sek-1 (km4)*. S*ek-1 (km4)* animals carry a mutation in the MAPK kinase pathway which leads to the hypersusceptibility of this *C.elegans* strain to Cry5B (27-29). Representative results of one of three independent trials are shown in Figure 7. In Figure 7 A, *sek-1 (km4)* worms treated with host strain MG1363 showed no effect of the added bacterial cell lysate. Similarly, panels B and D with lysates from MG1363 with the constitutive vector (pTRK593) and constitutively expressed tCry5B (pTRK1041) did not show an effect. However, cell lysates from cells constitutively expressing Cry5B (pTRK1040, panel 7C) were clearly intoxicating to the worms. Cell lysate from MG1363 containing the nisin inducible empty vector (pMSP3535H3) also had an effect on the *sek-1 (km4) C. elegans*, likely due to the worms' sensitivity to nisin, which was added to the culture of the *L. lactis* control strain prior to cell lysate preparation. However, there was a clear additional effect of both nisin-induced Cry5B (from pTRK1068, panel F) and also nisin-induced tCry5B (from pTRK1069, panel G). These assays demonstrate striking biological activity of both Cry5B and tCry5B in the *sek-1 (km4) C. elegans* strain. In addition, these results suggest biological activity from the processed version of full length Cry5B shown in the immunoblot in Figure 3 (120 kD compared with unprocessed

Cry5B (140 kD), since most of the nisin-induced Cry5B from pTRK1068 is in the processed form.

Discussion

In this study we demonstrate that *B. thuringiensis* Cry protein Cry5B (140 kDa) and a truncated form (tCry5B, 79 kDa) can be expressed intracellularly and released extracellularly in *L. lactis*. This is a first step towards the use of *L. lactis* as a delivery vehicle for Cry5B in the treatment of mammalian helminth infections.

New anthelmintics must be inexpensive and produced in large quantities for delivery under adverse environmental conditions (4). Ideally, they will not require cold storage and could be delivered in a single dose. *L. lactis* is a food-safe, GRAS organism, which can be engineered to deliver Cry proteins safely and economically for treatment of helminths.

In this study, Cry5B was overexpressed using nisin induction. Nisin is an antimicrobial peptide secreted by various strains of *L. lactis*, which can be used as a natural preservative. Nisin induction has been widely used in *L. lactis* and other bacteria for expression of heterologous proteins, and is described as simple to use with high yields (11). The *L. lactis* nisin operon consists of 11 genes. Among these, the NisA protein is the active peptide, and its production is controlled by NisR, a regulator protein, and NisK, a membrane kinase which is activated in the presence of nisin (37). NisI, appears to act cooperatively with NisFEG to provide cellular immunity to nisin (37). The *nisI*, *nisR* and *nisK* genes are present in pMSP3535H3, the expression vector used in this work. NisI provides enough added resistance to nisin to enable

348 growth in up to 200 ng/ml nisin (34). Cry5B was strongly induced by nisin compared to the base
349 level of expression from strong constitutive promoter encoded on pTRK593. However most of
350 the nisin-induced Cry5B protein was reduced in size from 139 kD to about 120 kD. The reason
351 for this clipping is unknown. Nevertheless, biological assays with *C. elegans sek-1 (km4)*
352 demonstrated that the nisin-induced Cry5B and tCry5B effectively halted worm development.

353 Cytoplasmic proteins without signal sequences are commonly found in cell supernatants due to
354 cell lysis. Intriguingly, a study of cytoplasmic proteins found in *Staphylococcus aureus* cell
355 supernatants was done by comparing wild type cells with mutants in the major autolysin Atl (38).
356 It was found that the presence or absence of prophages had little effect on the secretome of *S.*
357 *aureus*. Twenty-two cytoplasmic proteins which were present in the secretome of the wild type
358 were significantly decreased in the *atl* mutant, confirming a role for the autolysin in release of
359 the proteins. A selection mechanism in the excretion of cytoplasmic proteins was postulated but
360 not characterized. *Lactococcus lactis* is known to exhibit extensive autolysis capability (39). It is
361 possible that low level expression of an autolysin enables externalization of Cry5B when
362 expressed constitutively from pTRK1040 and pTRK1041 in *L. lactis*.

363 The leaky *Lactococcus* system used here to externalize Cry5B and tCry5B depends on the
364 constitutive activation of a prophage holin and lysin cassette by a transcriptional activator,
365 Tac31A, on a high copy number plasmid. In the original characterization of the system, up to
366 88% of the β-galactosidase (β-gal) activity of *L. lactis* KP1 was detected in the cell supernatants,
367 with no discernible effect on cell growth, lysis, or membrane integrity (24). The leaky system,
368 like the *S. aureus* autolysin-controlled system (38) is selective. While β-gal (117 kDa) and
369 tetanus toxin fragment C (47 kDa) were externalized from the leaky system, PepXP (176 kDa)

370 five other peptidases (30-100 kDa) were not (24, 40). None of the peptidases, PepA, PepC,
371 PepN, PepO, and PepXP, have transmembrane helices or signalP sequences, suggesting that all
372 are cytosolic. The determining factor in whether a protein is externalized is unknown. Protein
373 mass of the tested proteins (as monomers) is evidently not a factor. Remarkably, in this work,
374 virtually all of the cytoplasmic Cry5B (140 kDa) and tCry5B (79 kDa) proteins are externalized
375 by the leaky system, with no effect on cell growth. Cell membrane permeability appears to be
376 unaffected in log phase leaky cells, but the leaky phenotype appears to cause considerable
377 permeability in stationary phase cells.

378 The Tac31A activator regulates a late phage promoter of a prophage in *L. lactis* KP1(24). While
379 *L. lactis* MG1363 encodes prophage sequences, β-gal was not externalized from this strain,
380 suggesting that Tac31A is not an activator of a holin/lysin promoter for this strain (24, 41).
381 Investigation of recombinant phages arising from KP1 has indicated that there are two distinct
382 regions of the KP1 genome which can contribute to recombinant phages, and therefore must
383 contain prophage sequences (42). KP1 holin and lysin genes were not identified in the
384 recombinant phages, which arose in response to a phage resistance mechanism which targeted
385 phage replication. One of the temperate phages can be induced from KP1 using mitomycin C,
386 and closely resembles temperate phages of two other lysogenic *L. lactis* strains. (43). Activation
387 of the late prophage transcript(s) is weak, allowing the cells to continue to grow (24). Further
388 investigation will be required to determine the mechanism of cytosolic protein release by the
389 leaky *Lactococcus* system.

390 In vivo challenges to *C. elegans* with cell lysates of *L. lactis* expressing Cry5B and tCry5B
391 demonstrated biological activity and abated worm development. Nisin-induced expression of

both Cry toxins showed distinct biological activity, in synergism with or in addition to the effect of nisin, whereas only Cry5B showed activity expressed from the base constitutive expression plasmid, pTRK593.

We have shown here that active full length and truncated Bt Cry5B and tCry5B proteins can be expressed in *L. lactis* cells, both constitutively and through nisin induction. Both forms of Cry5B are found intracellularly and in culture supernatants from log phase *L. lactis* MG1363 and KP1 cells when expressed constitutively. The externalization of Cry5B occurs without any detectable disruption in cell growth or membrane integrity, and in the absence of increased cell death. After nisin induction in log phase *L. lactis* MG1363, increasing amounts of Cry5B are detected intracellularly with increasing concentrations of nisin. However Cry5B is not detected in the cell supernatants, perhaps because the time period of the 3 h assay is not long enough to allow for externalization. When Cry5B is expressed constitutively in *L. lactis* KP1 using the leaky lactococcus system, virtually all of the protein was found in the cell supernatants, and none detected intracellularly. Together, these systems provide multiple options for expression of Cry5B and tCry5B active proteins from *L. lactis* for possible use as an oral anthelminthic.

Acknowledgements

This work was supported by The Bill & Melinda Gates Foundation Grand Challenges Exploration (grants OPP1025524 and OPP1067992) and the North Carolina Agricultural Foundation. The authors thank Sarah O'Flaherty, Y. Jun Goh, and Debra Clare for helpful suggestions and discussions. We thank David Mills for providing plasmid pMSP3535H3.

Figure Legends

Fig. 1. Immunoblot showing Cry5B and truncated Cry5B (tCry5B) expression in log phase cultures constitutively (vector, pTRK593; Cry5B, pTRK1040; tCry5B, pTRK1041) in *E. coli* and *L. lactis* MG1363 and KP1; and from leaky vector (pTRK1061) in *L. lactis* KP1 (Cry5B, pTRK1062; tCry5B, pTRK1063). Panel A. Intracellular, cell lysates. Panel B. Extracellular, cell free supernatants.

Fig. 2. Effect of added nisin on *L. lactis* MG1363. Panel A. Growth curves after the addition of nisin to log phase cultures at approximately OD_{600} 0.4 showing the *Lactococcus* host MG1363 parent strain (nisin sensitive), MG1363 harboring the base nisin inducible vector (pMSP3535H3); and *Lactococcus lactis* ATCC 11454, a nisin producing, naturally nisin-resistant *L. lactis* strain. Averages of three independent experiments are shown. Panel B. LIVE/DEAD BacLight assay showing the effects of increasing nisin concentrations on the membrane permeability of *L. lactis* MG1363 cells transformed with either the nisin inducible vector (pMSP3535H3) or the vector with the full length Cry5B insert (pTRK1068).

Fig. 3. Nisin induction of Cry5B expression from *Lactococcus lactis*. Controls show *L. lactis* MG1363 and KP1with vector (pTRK593) and constitutive expression of Cry5B (pTRK1040). Nisin induced expression of Cry5B is from MG1363 with pTRK1068, at increasing concentrations of nisin, indicated by triangles. Samples were loaded at different protein concentrations shown as 2.5, 5 or 10 µg protein per lane).

433 Fig. 4. Growth curves for *L. lactis* KP1 with base constitutive expression plasmid vector
434 (pTRK593), constitutive Cry5B expression (pTRK1040), base leaky vector (pTRK1061) and
435 leaky Cry5B expression (pTRK1062).

436 Fig. 5. Representative Live/Dead assays using the BacLight kit showing membrane permeability
437 of A. log phase cells and B. overnight/stationary *L. lactis* cells: MG1363, KP1, and KP1 with the
438 leaky vector (pTRK1061) and leaky expression of Cry5B (pTRK1062).

439 Fig. 6. LDH enzyme activity assays of supernatants from log phase *L. lactis* cells in three
440 experiments. LDH enzyme (5 U) was used as a positive control. Constitutive (constit.) vector is
441 pTRK593; constitutive Cry5B and truncated Cry5B (tCry5B) strains contain pTRK1040 and
442 pTRK1041 respectively; the leaky strains contain vector plasmid pTRK1061; leaky Cry5B,
443 pTRK1062; and leaky truncated Cry5B (tCry5B), pTRK1063.

444 Figure 7: Biological activity assays of Cry5B proteins expressed from *L. lactis. C. elegans sek-*
445 *1 (km4)* worms were challenged with cell lysates of *L. lactis* MG1363 producing Cry5B or
446 tCry5b from constitutive or nisin-inducible expression plasmids. Panel A, host MG1363; Panel
447 B, constitutive expression vector only pTRK593; Panel C, constitutively expressed Cry5B in
448 pTRK593 (pTRK1040); Panel D, constitutively expressed tCry5B in pTRK593 (pTRK1041);
449 Panel E, base nisin-inducible plasmid (pMPS3535H3); Panel F, nisin-induced Cry5B in
450 pMPS3535H3 (pTRK1068); Panel G, nisin induced tCry5B in pMPS3535H3 (pTRK1069).

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454 Table 1. Bacterial strains and plasmids used in this work

Bacterial strain or plasmid	Description	Source or reference
Strains		
Escherichia coli DH5α	cloning host	Invitrogen
Escherichia coli XL1-Blue	cloning host	Stratagene
Lactococcus lactis MG1363	expression host; plasmid cured	(41, 44)
Lactococcus lactis KP1 (NCK203)	expression host; 2 native plasmids, and 2 prophages	(45)
Lactococcus lactis subsp. *lactis* ATCC 11454	Nisin producer and nisin resistant *L. lactis* strain	ATCC*
Plasmids		
pQE9 Cry5B	Source of *cry5B* gene for cloning	(46)
pSC-B	PCR cloning vector	Agilent Technologies
pTRK593	pTRKH2** based cloning vector with P6 promoter	(23)
pTRK1040	Bt Cry protein Cry5B cloned into pTRK593	This study
pTRK1041	Bt Cry protein tCry5B cloned into pTRK593	This study

pTRK1061	pTRKH2 based expression vector with leaky system promoter and activator	This study
pTRK1062	Bt Cry protein Cry5B cloned into pTRK1061	This study
pTRK1063	Bt Cry protein tCry5B cloned into pTRK1061	This study
pMSP3535H3	nisin expression vector with nisin and erythromycin resistance	(34)
pTRK1068	Bt Cry protein Cry5B cloned into pMSP3535H3	This study
pTRK1069	Bt Cry protein tCry5B cloned into pMSP3535H3	This study

455

456 * American Type Culture Collection, Manassas, VA.

457 **pTRKH2 is a 6.9 kb high copy number *E. coli*/gram positive shuttle vector encoding

458 Erythromycin resistance (32).

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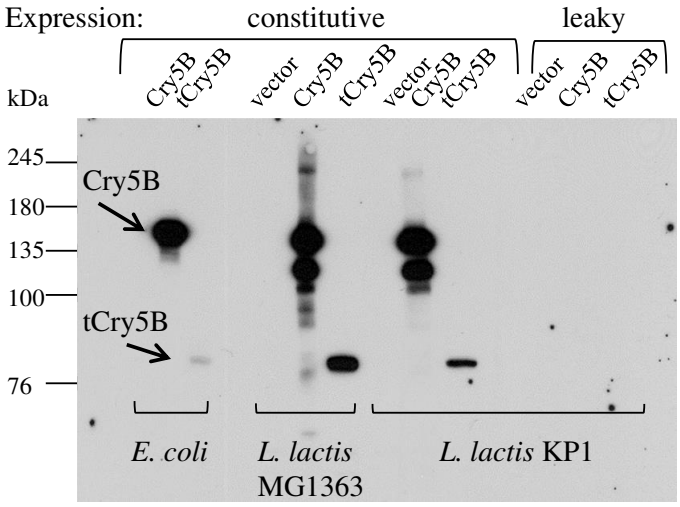

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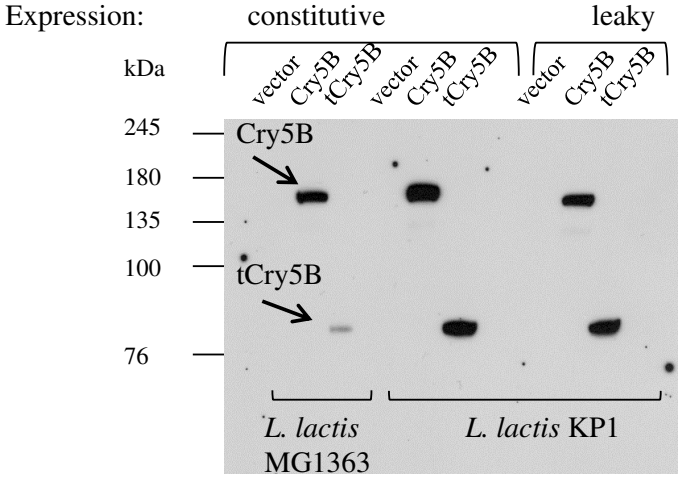

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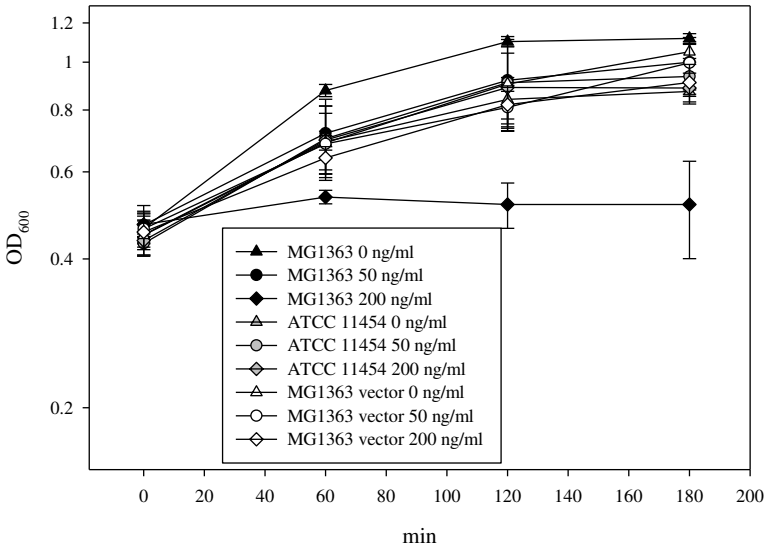

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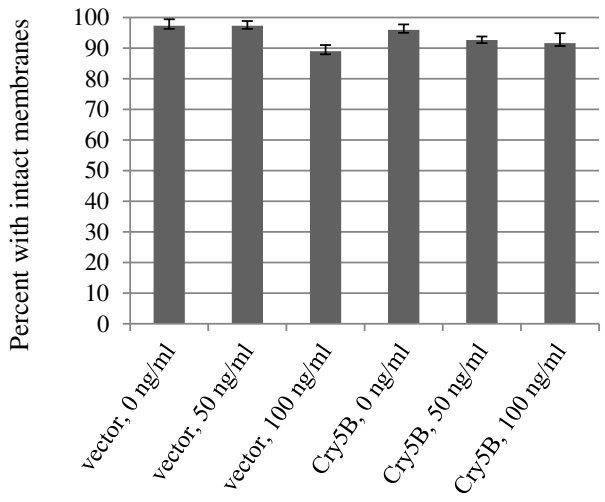

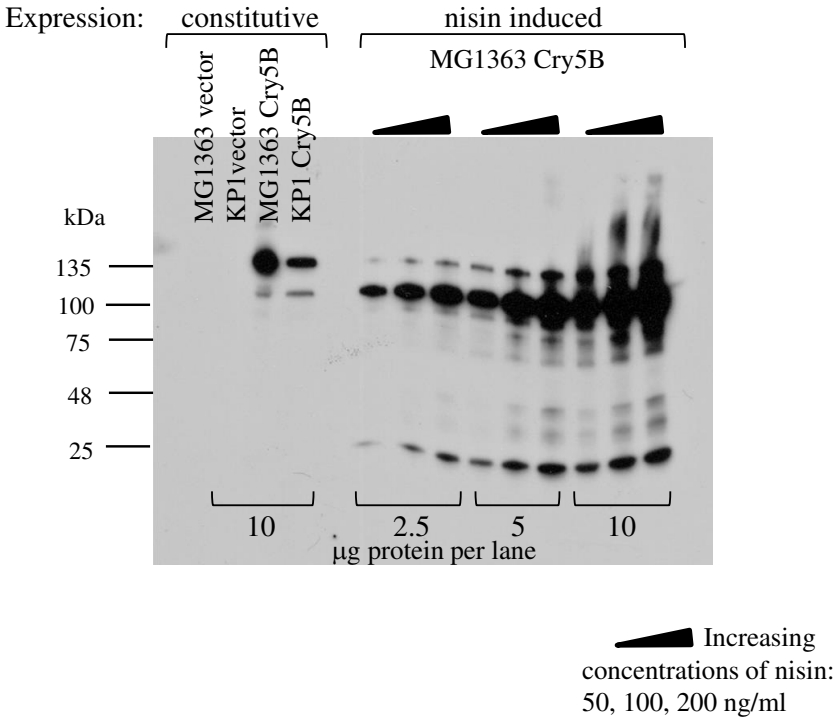

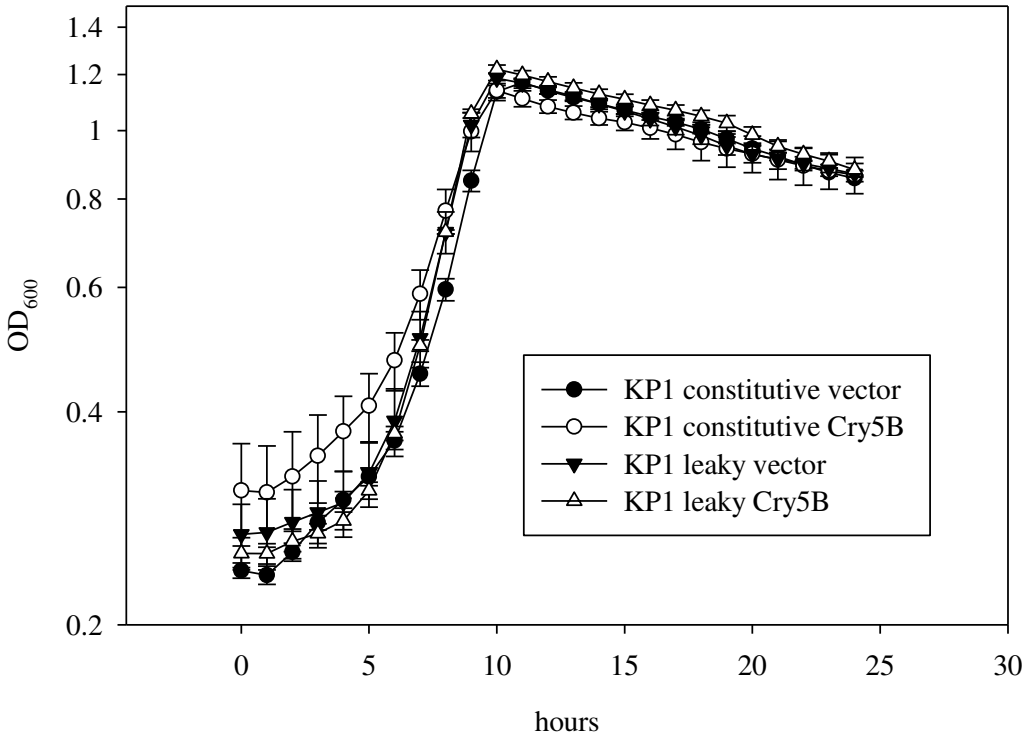

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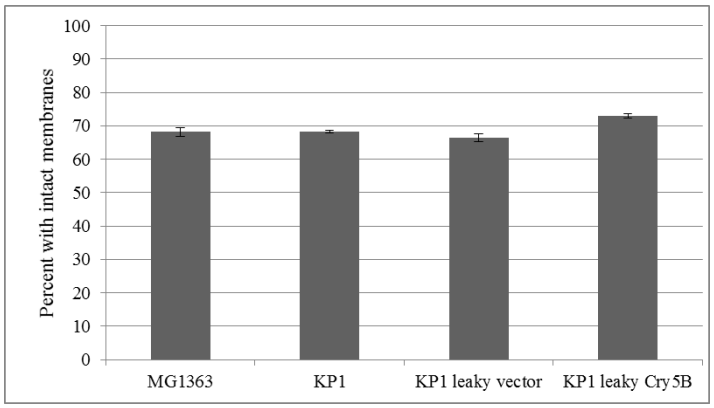

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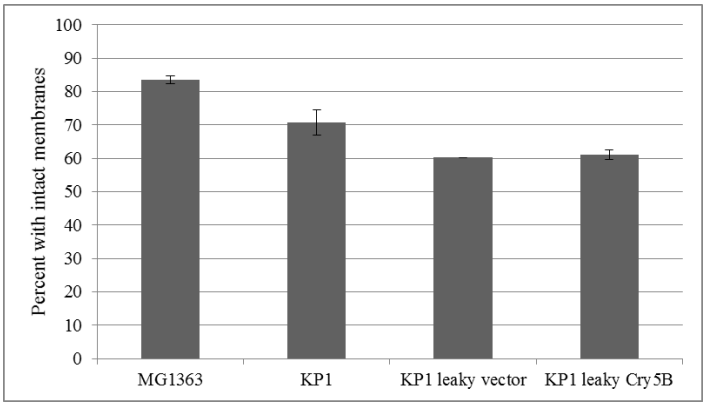

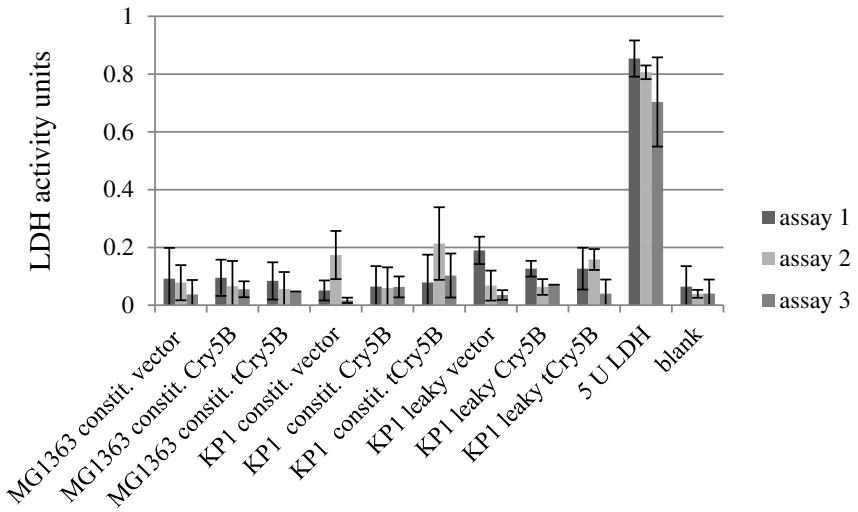

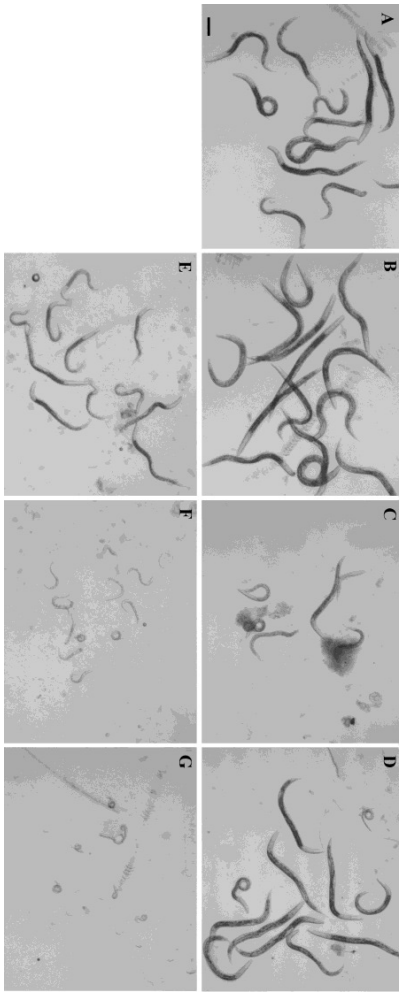
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