Components of the Cultivated Red Seaweed Chondrus crispus Enhance the Immune Response of Caenorhabditis elegans to Pseudomonas aeruginosa through the pmk-1, daf-2/daf-16, and skn-1 Pathways

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Marine macroalgae are rich in bioactive compounds that can, when consumed, impart beneficial effects on animal and human health. The red seaweed Chondrus crispus has been reported to have a wide range of health-promoting activities, such as antitumor and antiviral activities. Using a Caenorhabditis elegans infection model, we show that C. crispus water extract (CCWE) enhances host immunity and suppresses the expression of quorum sensing (QS) and the virulence factors of Pseudomonas aeruginosa (strain PA14). Supplementation of nematode growth medium with CCWE induced the expression of C. elegans innate immune genes, such as rgl-1, rgl-2, F49F1.6, bsf-1, K05D8.5, F56D6.2, C29F3.7, F28D1.3, F38A1.5 ZK6.7, lys-1, spp-1, and abf-1, by more than 2-fold, while T20G5.7 was not affected. Additionally, CCWE suppressed the expression of PA14 QS genes and virulence factors, although it did not affect the growth of the bacteria. These effects correlated with a 28% reduction in the PA14-inflicted killing of C. elegans. Kappa-carrageenan (K-CGN), a major component of CCWE, was shown to play an important role in the enhancement of host immunity. Using C. elegans mutants, we identified that pmk-1, daf-2/16, and skn-1 are essential in the K-CGN-induced host immune response. In view of the conservation of innate immune pathways between C. elegans and humans, the results of this study suggest that water-soluble components of C. crispus may also play a health-promoting role in higher animals and humans.

Seaweeds are rich in bioactive compounds, such as proteins, peptides, amino acids, lipids, fibers, pigments, polyphenols, and polysaccharides (1, 2), that are responsible for imparting various health benefits. For example, β-carotene and lutein were identified as antimutagenic substances in edible red algae, which indicated their potential anticancer activity (3). Furthermore, studies on mice/rats and humans demonstrated that dietary supplementation with various extracts of a variety of seaweeds correlated with a decreased risk of breast cancer (4, 5). Polysaccharides, proteins, peptides, and amino acids from a number of seaweeds showed beneficial activity against diabetes, cancer, AIDS, and vascular diseases (2). The red seaweed Chondrus crispus (Rhodophyta) is widely distributed in the northern Atlantic. The work presented here was undertaken using a proprietary strain of C. crispus which was cultivated on land in Nova Scotia, Canada, for the Asian food market by Acadian Seaplants Limited. Besides high contents of total proteins, oligopeptides, and pigments, this red alga is rich in the water-soluble polysaccharide carrageenan (CGN) (6), which has been reported to have antiviral (7, 8) and antitumor (9, 10) activities.

CGNs are linear polymers of digalactose residues and can be extracted from some species of red seaweed. CGNs are widely used in the food industry as thickeners, stabilizers, and emulsifiers. C. crispus produces three types of CGN at different stages of its life cycle. In the diploid sporophyte phase, it produces lambda-CGN, while the haploid gametophyte produces predominantly kappa-CGN (K-CGN) with some iota-CGN. The gametophyte also makes the precursor types of kappa- and iota-CGNs, mu- and nu-CGNs. The mu- and nu-CGNs are more sulfated than the kappa- and iota-CGN types, and they are of nongelling forms. These precursors are more similar to lambda-CGN with respect to sulfation levels and solubility properties (J. S. Craigie, personal communication). The water extract used in the present work contained multiple compounds, with K-CGN being the main type of CGN.

The nematode (Caenorhabditis elegans) model has been used to study fundamental biological processes and also for the rapid screening of chemical compounds for various health effects. In the present work, the C. elegans model of infection with Pseudomonas aeruginosa was used to investigate the immune-enhancing activity of a cultivated C. crispus water extract (CCWE). The ubiquitous bacterium P. aeruginosa is an emerging opportunistic human pathogen which infects immunodeficient or immunocompromised patients (11). It also causes lethal infection of the nematode C. elegans (12). The pathogenesis of P. aeruginosa is mediated by secreted virulence factors, which include toxins, such as pyocyanin, pyoverdine, siderophores, and hydrogen cyanide (12, 13), as well as by bacterial enzymes, such as elastase and alkaline protease (14). Moreover, biofilm formation protects the bacteria from adverse environmental factors and increases their antibiotic resistance and pathogenesis (15). Interestingly, the virulence factors and biofilm formation were found to be regulated by quorum
sensing (QS), the cell-to-cell communication system of the bacteria. The two QS systems include the lasR-lasI and the rhlR-rhlI systems, where lasR and rhlR are transcription activators and lasI and rhlI are the synthases of small interactive autoinducer molecules (14, 16).

The immune response of C. elegans to P. aeruginosa is mediated through the p38 mitogen-activated protein kinase (PMK-1), transforming growth factor β (TGF-β), and the DAF-2/DAF-16 insulin-like and ZIP-2 pathways (17–20). Recently, an extract of the brown seaweed Ascosiphon nodosum was demonstrated to protect C. elegans from infection by P. aeruginosa strain PA14 (21). In the present study, we tested the effects of a water extract from the cultivated red seaweed C. crispus on host immunity and PA14 pathogenicity using the C. elegans infection model with PA14. We further examined the effect of pure K-CGN, the predominant water-soluble polysaccharide present in C. crispus, on host immunity. Moreover, we utilized a number of mutant lines of C. elegans to determine the role of various signaling pathways in the K-CGN-elicited immune response.

MATERIALS AND METHODS

Preparation of seaweed extracts. The on-land-cultivated proprietary strain of C. crispus used in this study was obtained from Acadian Seaplants Limited (Dartmouth, NS, Canada). The dried seaweed (250 g) was extracted with water (two times with 1.5 liters each time) by sonication for 1 h at room temperature. The aqueous fraction was concentrated under reduced pressure and freeze-dried overnight, yielding water extract (CCWE; 67.1 g). The CCWE was stored at −20°C. Research-grade K-CGN powder was obtained from Cargill Texturant Systems, France. A stock solution of CCWE or K-CGN was prepared by dissolving the powder in distilled water to a concentration of 25 mg/ml or 10 mg/ml, respectively.

Preparation of A. nodosum. The two QS systems include the synthases of small interactive autoinducer molecules (17–20). Recently, an extract of the brown seaweed Ascosiphon nodosum was demonstrated to protect C. elegans from infection by P. aeruginosa strain PA14 (21). In the present study, we tested the effects of a water extract from the cultivated red seaweed C. crispus on host immunity and PA14 pathogenicity using the C. elegans infection model with PA14. We further examined the effect of pure K-CGN, the predominant water-soluble polysaccharide present in C. crispus, on host immunity. Moreover, we utilized a number of mutant lines of C. elegans to determine the role of various signaling pathways in the K-CGN-elicited immune response.

Infection experiments and gene expression analysis of C. elegans and PA14. Pathogen infection experiments were performed as described above for the killing assay, except that approximately 100 worms were transferred to each plate and harvested at 3 h, 6 h, 24 h, or 48 h postexposure to the PA14 lawn. The worms were washed 3 times in M9 buffer to eliminate excess bacteria. The tubes with worms were briefly chilled on ice to acquire stacked worms, and the buffer was removed by pipetting. Prior to the gene expression analysis of PA14, bacteria with an initial OD600 of 0.02 were cultured at 37°C in KB broth in the presence (treatment) or absence (control) of 500 μg/ml CCWE with constant shaking. The overnight culture was centrifuged for 10 min at 1,500 × g to pellet the bacteria. Total RNA was extracted from stacked worms or bacterial pellets with TRIzol reagent (Invitrogen) and an RNeasy RNA kit (Qiagen) following the manufacturer’s protocol. The integrity and quantity of the RNA were assessed by agarose gel electrophoresis and with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). For gene expression analysis, RNA samples derived from three biological replicates of each treatment were pooled. From 2 μg of total RNA, cDNA was synthesized using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was performed on a StepOne real-time PCR system (Applied Biosystems) using Promega GoTaQ SYBR green reagent (Roche Diagnostics, Mississauga, ON, Canada) with 0.2 μM each gene-specific primer and 10 ng of cDNA as the template. Each pooled sample, representing three biological replicates, was run in a reaction mixture with a final volume of 10 μl in triplicate, following the manufacturer’s instructions. For gene expression analysis of C. elegans, ama-1 or nhr-23, whichever was more stably expressed in the specific sample sets, was used as an endogenous control. For PA14 gene expression, 16S rRNA served as an endogenous control. The sequences of the primers are listed in Table S2 in the supplemental material.

Protease assay. PA14 was cultured in the presence (treatment) or absence (control) of 500 μg/ml of CCWE, and protease activity was determined spectrophotometrically at 600 nm by measurement of the skim milk hydrolysis efficacy of the protease secreted in the culture. The protease activity was expressed as mg of protein hydrolyzed per ml culture per hour, using a standard curve generated from a serial dilution of skim milk (skim milk powder for microbiology; BDH) (23).
Alkaline protease assay. The alkaline protease activity of the supernatant of PA14, cultured in the presence (treatment) or absence (control) of 500 μg/ml of CCWE, was assayed using elastin-Congo red powder (Sigma). The alkaline protease activity was expressed as units where 1 unit was defined as an increase of 1.0 in the OD590 per ml per hour (24).

Elastase assay. The elastase activity of PA14, cultured in the presence (treatment) or absence (control) of 500 μg/ml of CCWE, was determined using elastin-Congo red powder (Sigma). Elastase activity was expressed as the increase in the OD455 per ml of PA14 filtrate (25).

HCN assay. Hydrogen cyanide (HCN) production by PA14 in the presence (treatment) or absence (control) of 500 μg/ml of CCWE was visualized by the changing color of sodium picrate due to the reduction activity of HCN (26). Filter paper disks were saturated with sodium picrate and attached onto the lower side of the lid of culture plates which were seeded with PA14. The plates were sealed and incubated prior to elution, and the brownish yellow compound on the discs was spectrophotometrically quantified. The quantity of the corresponding HCN was expressed as the OD625 (27).

Pyocyanin assay. Pyocyanin in 3 ml of filtrate of PA14, cultured in the presence or absence of 500 μg/ml of CCWE, was extracted with chloroform-HCl and quantified by determination of the absorbance at 520 nm, as previously described (28), with minor modifications.

Siderophore assay. The PA14 siderophore in a 20-ml culture filtrate with (treatment) or without (control) 500 μg/ml of CCWE was extracted in ethyl acetate, dried with a nitrogen evaporator (N-EVAP; Efanol Research Inc., Canada), dissolved in ethanol, mixed with Hathway’s reagent, and subjected to reading of the absorbance at 700 nm. The quantity of siderophore was expressed as the OD700 of the culture filtrate (29).

Microtiter plate biofilm assay. The relative quantity of biofilm was determined by reading the OD590 on a microplate reader (BioTek) (30). The PA14 biofilm which was formed in the presence (treatment) or absence (control) of 500 μg/ml of CCWE was stained with crystal violet and quantified by determination of the absorbance at 520 nm, respectively; K-CGN200, 200 μg/ml of kappa-carrageenan. The purity of the K-CGN sample used in the present work was confirmed by proton nuclear magnetic resonance on a Bruker 700-MHz spectrometer (see Fig. S2 in the supplemental material). The statistical analyses were performed using SPSS software, version 15.0. The comparison of survival curve data was carried out using the log-rank test of the Kaplan-Meier survival function, while an independent t test was applied to the analysis of the other data. Differences were considered significant when P was <0.05.

RESULTS

CCWE and K-CGN protect C. elegans from PA14 infection. Wild-type C. elegans N2 worms were cultured with CCWE as a food supplement from the early L1 stage. On day 1 of adulthood, the worms were exposed to PA14 infection in the presence or absence of CCWE in the culture medium. The survival rate of the worms was recorded every 24 h. Worms cultured without CCWE supplementation, or plain worms, followed by plain PA14 (PA14 cultured without CCWE) exposure, served as the control. It was found that CCWE treatment resulted in survival rates higher than those for the control when CCWE was used either as a worm food supplement (P < 0.0001) (see Fig. S1A and Table S1 in the supplemental material) or as an inhibitor of PA14 pathogenicity in the culture medium (P < 0.0001) (see Fig. S1B and Table S1 in the supplemental material). Interestingly, the combination of the two treatments protected a larger fraction of the worms from bacterial infection (Fig. 1 and Table 1). Specifically, all untreated worms died at 120 h, while 500 μg/ml of CCWE protected up to 28% of the worms against the lethal effect of PA14 infection (P < 0.0001). Although the treatment with 750 μg/ml of CCWE as a food supplement resulted in a better survival rate than treatment with 500 μg/ml of CCWE as a food supplement (see Fig. S1A in the supplemental material), 250 or 750 μg/ml of CCWE showed less efficacy for protection in both the inhibition of PA14 pathogenicity (see Fig. S1B in the supplemental material) and the synergism (Fig. 1) experiments. CCWE at concentrations between 250 and 750 μg/ml did not negatively affect the growth or development of the animal (data not shown). On the basis of these observations, 500 μg/ml was identified as the optimum CCWE concentration for protection of the worms from the lethal effect of PA14 infection; therefore, this dosage was used in all further assays.

The components of CCWE were investigated for their effects on host immunity induction. On the basis of the previously reported immune-modulating effects of CGN and the fact that CGN contributed 40% of the weight of our CCWE preparation (31), we reasoned that the predominant water-soluble polysaccharide in CCWE, K-CGN, might have played a role in the observed protection of C. elegans against PA14 infection. Consequently, the effect of pure K-CGN was tested using the C. elegans infection model. The purity of the K-CGN sample used in the present work was confirmed by proton nuclear magnetic resonance on a Bruker 700-MHz spectrometer (see Fig. S2 in the supplemental material). To match the optimal concentration of CCWE, 200 μg/ml of K-CGN was used. The protective effects of K-CGN observed were similar to the effects previously observed with CCWE (Fig. 1 and Table 1).
against PA14. C07G3.2 included 7 early response genes, i.e., noninfected worms were analyzed by qPCR. The selected genes for 1), (ShK domain-like, secreted surface protein), which immune pathway was important for the K-CGN-induced protection against lethal infection with PA14. As shown in Fig. 2, which immune genes were significantly activated by PA14 infection; on the other hand, the genes were not further activated by CCWE, except for irg-1, irg-2, and lys-1 (Fig. 3A and B). On the contrary, at 24 h postinfection, except for C29F3.7, F28D1.3, and ZK6.7, all genes were suppressed in PA14-infected worms compared to their expression in the control worms fed OP50. Notably, at this time point, CCWE treatment of either worms or PA14, or both worms and PA14, resulted in a significant activation of all 14 immune genes in PA14-infected worms. The CCWE-activated genes with more than 2-fold changes under infection conditions were as follows: irg-1, irg-2, F49F1.6, hsf-1, F56D6.2, C29F3.7, F28D1.3, ZK6.7, lys-1, spp-1, and abf-1. The expression of K05D8.5, T20G5.7, and F38A1.5 was apparently less affected by CCWE (P < 0.01) (Fig. 3C and D). Overall, CCWE enhanced immunity in C. elegans. K-CGN activated most of the immune genes tested under both infection and noninfection conditions at both time points (6 h and 24 h) (see Fig. S5 in the supplemental material), in a pattern similar to that of CCWE treatment.

CCWE suppresses expression of quorum-sensing and virulence factor genes of PA14. Besides the enhanced immunity of the host, another mechanism which may act to attenuate the death of worms following PA14 exposure is the inhibition of virulence factors by CCWE treatment. Therefore, the effect of CCWE on the expression of the quorum-sensing genes and the virulence factor genes of PA14 was investigated. CCWE repressed the expression of a number of QS and virulence factor genes in PA14, without affecting its housekeeping genes (Fig. 4). The QS genes, namely, lasI, lasR, rhlI, and rhlR, were repressed by 4- to 20-fold (P < 0.0001). Likewise, virulence factor genes, including hcnC, aroE, rpoN, sbe, and sodB, were downregulated by approximately 2- to 50-fold with treatment with 500 μg/ml of CCWE in the culture medium (P < 0.01).

Biochemical evidence for inhibition of the virulence factors of PA14 by CCWE. CCWE significantly downregulated PA14 virulence factor genes; therefore, quantification of PA14 virulence factors was undertaken. Biochemical assays demonstrated an overall reduction of the virulence factors by CCWE treatment (P < 0.01) (Fig. 5). The activity of the pathogen-secreted enzyme total protease was significantly inhibited by CCWE treatment (P = 0.001), while the alkaline protease activity was not apparently affected (P = 0.07) (Fig. 5A and B). Another PA14-secreted enzyme, elastase, showed a 3-fold decrease in activity with CCWE treatment (P < 0.0001) (Fig. 5C). Additionally, all 4 PA14-se-
creted toxins were significantly reduced ($P < 0.01$) (Fig. 5D to F). Specifically, in the presence of CCWE in the culture medium, PA14 produced 30% less HCN than the control, which was visualized by the lighter color of the sodium picrate-saturated filter paper in the CCWE-treated PA14 plates ($P < 0.001$) (Fig. 5D). In addition, pyocyanin production was reduced by as much as 45% in CCWE-treated PA14 ($P < 0.0001$) (Fig. 5E). Similarly, PA14 secretion of siderophore was reduced by 25% by CCWE treatment ($P < 0.0001$) (Fig. 5F).

**DISCUSSION**

The clinical isolate *P. aeruginosa* PA14 is pathogenic to both *Arabidopsis thaliana* and severely burned mice, with PA14 infection in the latter case being lethal (34). The pathogenicity in both the plant and animal models was mediated by a number of virulence factors, including *toxA*, *plcS*, and *gacA*. Interestingly, PA14 and another strain of *P. aeruginosa*, PAO1, were demonstrated to kill the soil nematode *C. elegans* in either hours or days, depending on the type of growth medium used in the killing assay (12, 35). In the present study, we utilized the *C. elegans* infection model to investigate the effects of a water extract from a commercially cultivated strain of the red seaweed *C. crispus* on both host immunity and *P. aeruginosa* pathogenicity. We observed that 500 μg/ml of CCWE...
exerted the best protection against PA14 killing for the worms, while higher or lower concentrations were less efficient. This result was consistent with what had previously been observed for a extract from the commercially harvested brown seaweed, A. nodosum, in a similar model system (21).

Thriving in coastal waters, seaweeds have evolved robust defense mechanisms, for example, inhibition of QS, against pathogens, such as the ubiquitous bacterium P. aeruginosa. Strikingly, CCWE presented properties as an inhibitor of QS in pathogenic strain PA14, which suggested that the red seaweed C. crispus per se can be of benefit for applications broader than just a functional food alone. In the growth curve assays, CCWE and its major component, K-CGN, did not abolish the growth of PA14 (see Fig. S6 in the supplemental material). In line with this, in a disk diffusion test, CCWE showed no direct antimicrobial activity (see Fig. S7 in the supplemental material). However, CCWE repressed the expression of PA14 QS genes and reduced the levels of PA14 virulence factors secreted, without affecting its housekeeping genes (see Fig. 4 and 5).

Another aspect of the C. elegans infection model was that the endpoint of PA14-inflicted killing for the control N2 worms was no earlier than 120 h postexposure, which was later than that described in previous reports (12, 38). An explanation for the slower killing can be the use of FUdR in the present study. FUdR blocks the midproliferation stage of the embryonic development of C. elegans (39), thus preventing eggs from hatching, which helped to avoid the confounding effects of progeny production. Immunity suppression in C. elegans was shown to be correlated with reproduction, or, more precisely, normal embryonic development. It was reported that sterile mutants of C. elegans, when placed under PA14 infection conditions, survived longer than wild-type N2 worms, and in accordance with this, FUdR-treated worms also survived longer (38).

C. elegans exhibited differential immune responses to PA14 at early (i.e., 6 h) and late (i.e., 24 h) stages of infection in this study. At 6 h postinfection, the majority of the tested immune genes were upregulated, while at 24 h postexposure to PA14, most genes were repressed. This finding was largely consistent with previous reports. For example, 4 h of exposure of C. elegans to PA14 resulted in activation of immune response genes, such as irg-1, irg-2, F49F1.6, K08D8.5, F56D6.2, and others (20). In addition, lys-1 and another two lysozyme genes, as well as the gene for a lipase, ZK6.7, were demonstrated to be inducible in C. elegans by the pathogenic bacterium Serratia marcescens (40). Similarly, infection of C. elegans by the Gram-positive bacterium Microbacterium nematophilus was correlated with the upregulation of a set of immune effectors, including, but not restricted to, F49F1.6, F56D6.2, and lysozymes (Lys3 and Lys7) (41). Despite the abundance of studies supporting immune induction by infection with a pathogenic organism, there is evidence showing immune suppression at a later time point of pathogen exposure. For instance, at 12 h of exposure to PA14, immune response genes such as spp-1, lys-7, and thn-2 were suppressed in C. elegans by activation of the DAF-2 insulin-like signaling pathway (33). Host immune suppression may represent one of the many strategies that the pathogen has evolved to counteract host immune defenses through a yet unknown mechanism (33, 42). However, research has shed light on both sides of the pathogen-host interaction: mounting of immune defense by the host and suppression of host immunity by the pathogen(s). It is possible that one side overcomes the other at certain time points during infection (32, 40, 41) and that the outcome of the battle can be regulated by external intervention. Indeed, in this study it was demonstrated at 24 h postexposure that a water extract of the commercially cultivated strain of the red seaweed C. crispus (CCWE) blocked the immune repression involving a majority of
the tested immune response genes in *C. elegans* induced by PA14. To better understand the function of these early and late immune response genes, we also examined their expression at 3 h and 48 h postexposure (see Fig. S8 in the supplemental material) and showed overall activation of the early immune response genes to PA14 infection and less activation of the late immune response genes. Strikingly, it is evident that CCWE enhances the immune activation or counteracts the immune suppression induced by pathogenic bacteria at various time points during the course of infection (Fig. 3; see Fig. S8 in the supplemental material). This is also associated with the suppression of QS and virulence genes, which contributes to an elevated rate of survival of the host. Surprisingly, K-CGN, a water-soluble polysaccharide present in *Chondrus*, induced a very similar pattern of host immune response in the N2 worms, which implies an important role for K-CGN in the CCWE-induced immunity. However, we did not observe a protective effect of K-CGN in the mutants, but a protective effect of CCWE cannot be ruled out, as it contains other bioactive components, albeit in less abundance.

Notably, in our present work, the selected immune genes were regulated by at least four distinct signaling pathways. For example, the infection response genes *irg-1* and *irg-2* are mediated by the *zip-2* pathway, while the ShK domain-like secreted surface protein *zip-2* transcription factor mediates an early response to infection (*Fig. 3; see Fig. S8 in the supplemental material*). This also associated with the suppression of QS and virulence genes, which contributes to an elevated rate of survival of the host. Surprisingly, K-CGN, a water-soluble polysaccharide present in *Chondrus*, induced a very similar pattern of host immune response in the N2 worms, which implies an important role for K-CGN in the CCWE-induced immunity. However, we did not observe a protective effect of K-CGN in the mutants, but a protective effect of CCWE cannot be ruled out, as it contains other bioactive components, albeit in less abundance.

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