Inhibitory effects of extracellular self-DNA: a general biological process?

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Summary

- Self-inhibition of growth has been observed in different organisms, but an underlying common mechanism has not been proposed so far. Recently, extracellular DNA has been reported as species-specific growth inhibitor in plants and proposed as an explanation of negative plant-soil feedback. In this work the effect of exDNA was tested on different species to assess the occurrence of such inhibition in organisms other than plants.
- Bioassays were performed on six species of different taxonomic groups, including bacteria, fungi, algae, plants, protozoa and insects. Treatments consisted in the addition to the growth substrate of conspecific and heterologous DNA at different concentration levels.
- Results showed that treatments with conspecific DNA always produced a concentration dependent growth inhibition, which instead was not observed in the case of heterologous DNA.
- Reported evidence suggests the generality of the observed phenomenon which opens new perspectives in the context of self-inhibition processes. Moreover, the existence of a general species-specific biological effect of exDNA raises interesting questions on its possible involvement in self-recognition mechanisms. Further investigation at molecular level will be required to unravel the specific functioning of the observed inhibitory effects.

Key words: autotoxicity, exDNA, self-recognition, exDNA functions, heterologous DNA.

Introduction

Self-inhibition or autotoxicity has been reported for several organisms including bacteria (Andersen et al. 1974; Trinick and Parker 1982), fungi (Bottone et al. 2011), algae (Inderjit and Dakshini 1994), plants (Singh et al. 1999) and animals (Akin 1966).

The mechanism has been mostly ascribed to the release and accumulation of different toxic compounds in the growth environment, but a specific class of chemicals accounting for both toxicity and species-specificity has never been identified. On the other hand, theoretical and modelling studies on species coexistence have suggested the involvement of a general mechanism to explain species-specific inhibition (Freitas and Fredrickson 1978; Bever 1994; Mazzoleni et al. 2010).

The recent observations by Mazzoleni et al. (2014) of inhibitory effects by extracellular self-DNA in plants provided new perspectives for understanding litter autotoxicity and negative plant-soil feedbacks. The authors reported significant evidence that fragmented extracellular DNA (exDNA) has a concentration dependent and species-specific inhibitory effect on plants' growth. These findings suggested an unexpected functional role of exDNA in intra- and inter-specific plant interactions at ecosystem level.

While the molecular mechanisms behind these phenomena certainly deserve in-depth investigations, more basic questions arise: does extracellular self-DNA act as inhibitor on biological systems other than plants? Could this be the general mechanism behind the observed phenomena of self-inhibition and autotoxicity?

Materials and Methods

In order to test the occurrence of species-specific inhibition by exDNA, a set of laboratory experiments was performed on six species selected across different taxonomic groups. Systematic experiments included exposures to self DNA and to heterologous DNA from *Arabidopsis thaliana* as a model organism, plus a control with distilled water. Extraction of genomic DNA from each species was performed using standard Qiagen® (Valencia, CA, USA) extraction kits and DNA purity was spectrophotometrically assessed at 260 nm on a NanoDrop TM 1000 (Thermo Scientific, Wilmington, DE, USA) and visually verified on 1.5% agarose gel using Sybr® Safe (Invitrogen). The extracted DNA was fragmented by sonication according to Mazzoleni et al. (2014) in order to obtain fragments mainly distributed in the range between 50 and 1000 bp, with similar size distribution for all DNA samples. The organisms were exposed to increasing concentrations of self-DNA while heterologous DNA was applied at the maximum concentration tested for self-DNA. Other experiments were preliminary performed to assess possible different effects from different sources of heterologous DNA. The specific experimental settings and treatment concentrations were adapted to the growth requirements of the different species as reported below. *Bacillus subtilis* was selected as target Gram-positive bacterium. It was pre-grown on Luria Broth (LB) at 37 °C with agitation (200 rpm). An inoculum was prepared with 10 ml of preculture and 4 ml of LB. Treatments included self-DNA at three concentration levels (40, 200, and 400 µg/ml) and heterologous DNA (400 µg/ml) from *A. thaliana*, *Aspergillus niger*, *Escherichia coli*, and *Sarcophaga carnaria*. All cultures were incubated with agitation (200 rpm) at 37 °C, with three replicates for each treatment and the control. After 24 hrs of incubation, 0.5 ml were taken from each tube and serial dilutions in LB were prepared, from which 100 µl were placed on LB agar plates. Plates were incubated at 37 °C until appearance of colony-forming units (CFU).

Trichoderma harzianum was used as target fungus in a bioassay on spore germination. Fungal spores were produced by pure cultures on potato dextrose agar (PDA). Spores were diluted to a concentration of 1 x 10^6 ml^{-1}. Treatments included extracellular self-DNA (8, 80, and 800 µg/ml) and heterologous DNA (800 µg/ml) from *A. thaliana*, *Aspergillus niger*, *Bacillus subtilis* and *Sarcophaga carnaria*, with three replicates for each treatment. The germination bioassay was performed in ELISA plates (96 wells, 100 µl each), each well coated with 10 µl of liquid 10% PDB substrate, DNA at treatment concentration, fungal spores, and sterile distilled water. Spore germination and germ tube elongation of the conidia were assessed by spectrophotometric analysis and optical microscopy after 20 hrs of incubation at 24 °C.

The green microalga *Scenedesmus obliquus* was maintained in Chu's n° 10 medium (Chu 1942). The cultures were incubated at 25°C under 270 µmoles photons m^{-2} sec^{-1} light intensity with 16:8 hrs light photoperiod. Treatments of *S. obliquus* were carried out with self-DNA (50 and 500 µg/ml) in the culture medium and heterologous DNA (500 µg/ml) from *A. thaliana*, with two replicates for each treatment. Algal growth was assessed by cell counts at the optical microscope after serial dilutions, and growth curves were built for each treatment, until reaching stationary phase (7 days).

Acanthus mollis seedlings were treated with self-DNA (2, 20, and 200 µg/ml) and heterologous DNA (200 µg/ml) from *A. thaliana*, *Quercus ilex* and *Sarcophaga carnaria*, with three replicates for each treatment. Bioassays were done in vitro by using surface sterile seeds (n=20 in each plate) placed in 9 cm Petri dishes over sterile filter papers imbibed with 4 ml of test solutions. Seedling root length was measured.

Plasmodia of the ameboid protozoan *Physarum polycephalum*, a slime mold widely used in bioassays were maintained in the dark at 24 °C on 1% agar plates and were fed with oat flakes. Laboratory stocks were subcultured onto new 1% water agar plates and fed oat flakes.

Mature cultures (15 days) on Petri plates were used to produce slime mold biomass for total DNA extraction. Tip portions (17±5 mm^2) of the plasmodia were taken from stock cultures 8 hours after feeding time and placed on agar substrates at the conditions of maintenance, with three replicated plates for each treatment and the untreated control. Extracted self-DNA (290, 580, and 1060 µg/ml) and heterologous DNA (1060 µg/ml) from *A. thaliana* were applied on 0.2 g of oat flakes placed at the centre of each plate. Pictures of plasmodial growth patterns were taken from each plate every 24 hrs for 96 hrs and used to calculate spreading area size following Takamatsu et al. (2009).

The dipteron *Sarcophaga carnaria* was grown in pure culture on 12 x 12 cm^2 plates (2 cm height) at 10 °C, fed with ground meat. Treatments included self-DNA (10, 100, and 1000 µg/ml) and heterologous DNA (1000 µg/ml) from *A. thaliana* mixed with 1 g of food. Three replicated plates, each containing 10 larvae, were prepared for each treatment, plus the untreated control. All plates were incubated in the dark at 10 °C. Development, survival, and time required for the formation of pupae were monitored every 3 days during a 21-days incubation period.

A generalized linear mixed model (GLMM) was used to analyse the results of the bioassays. Since different metrics were used to assess the performance of target species, data were expressed as percent of untreated controls. Tested effects on species performance included the target species (6 levels) as random effect, and treatment (3 levels: heterologous DNA, self-DNA and untreated control) and 2nd order interaction as fixed effects. Since the experimental design was not fully balanced with respect to concentration levels of DNA treatment, a further GLMM was tested to assess the effect of DNA concentration, limited to samples treated with self-DNA. Also in this model the target species (6 levels) and its interaction with self-DNA concentration were included as random effects. In both GLMMs pair-wise differences were tested for statistical significance using post-hoc Duncan tests.

Results

The experiments produced consistent results for all target species with evident effects of inhibition by self-DNA (Figure 1). The effect of all treatments was highly significant with different responses to either heterologous or self-DNA without differences between species (Table 1a). The application of heterologous DNA did not produce any significant growth reduction compared to control, with the exception of *B. subtilis* which showed some inhibition also in this case (Table 2). This was consistent with results from preliminary tests with different heterologous DNA sources, showing the absence of inhibitory effects in all cases, with the exception of the tested bacterium, which was inhibited at variable levels by heterologous DNA (Table 3).

On the contrary, treatments with conspecific DNA always resulted in a concentration dependent growth reduction (Table 1b), showing an inhibitory effect on all tested species (Table 2), consistent with the observations on plants by Mazzoleni et al. (2014). At lower self-DNA concentration the inhibitory effect was reduced with different responses for different species (see significant interactive term in Table 1b).

Discussion

Species-specific inhibitory effects of exDNA has been recently reported for higher plants (Mazzoleni et al. 2014). Here we extend such results to a set of organisms from different taxonomic groups.

Extracellular DNA has been found both in soil and marine sediments in large amounts (Steffan et al. 1988). Its long persistence in soil has been related to chemical stability and protection against enzymatic degradation by absorption to both mineral and organic components (Levy-Booth et al. 2007). Such accumulation of DNA molecules mainly derives from degradation of organic matter, though release by excretion from living cells is also reported (Nielsen et al. 2007).

Extracellular DNA has been proposed to serve different functions (Vlassov et al. 2007). It has been proposed to be a major source for the transfer of genetic information (Weinberg and Stotzky 1972; Graham and Istock 1978; Nielsen et al. 2007). It has been reported to play a role in the formation of microbial biofilms (Whitchurch et al. 2002; Steinberger and Holden 2005), in the protection from pathogen attack in root cap "slime" (Wen et al. 2009; Hawes et al. 2011) and in extracellular traps (Brinkmann et al. 2004; Goldmann and Medina 2012). Extracellular DNA has also been considered as a relevant source of nutrients for plants (Paungfoo-Lonhienne et al. 2010) and microbes (Finkel and Kolter 2001; Palchevskiy and Finkel 2006; Pinchuk et al. 2008).

The role of exDNA as species-specific inhibitor has been recently reported for higher plants (Mazzoleni et al. 2014), providing a novel explanation for negative plant-soil feedbacks such as inhibition of plant recruitment, growth and reproduction in soils previously occupied by conspecifics (Bever et al. 1997, van der Putten 2003; Kulmatiski et al. 2008; Mangan et al. 2010). The same effect could be the explanation of the frequently reported interspecific facilitation but rare occurrence of intraspecific facilitation in terrestrial ecosystems (Bonanomi et al. 2010). Further studies are needed to clarify the interplay between DNA persistence in the environment and related ecosystem diversity.

The experiments presented in this paper confirmed the occurrence and the concentration dependency of the inhibition by extracellular self-DNA in bacteria, fungi, algae, plants, protozoa and insects. The possible bias in these results by the presence of residual chemicals from DNA extraction can be excluded because the heterologous DNA, not producing inhibitory effects, was extracted with the same method and applied at the same high concentration of self-DNA.

The range of target species, including prokaryotes and both unicellular and multicellular eukaryotes, highlights the widespread occurrence of self-DNA inhibitory effect. An interesting evidence of self-inhibition in vertebrates was reported on *Rana pipiens* (Richards 1958, 1962), clearly showing a significant reduction of tadpoles growth in water previously occupied by conspecifics, unaffected by the presence of unrelated species and only slightly inhibited by phylogenetically related ones (Akin 1966). Richards (1958) suggested that "alga-like" pathogens could be the cause of the observed growth inhibition, but the involvement of such pathogens in small tadpoles inhibition was later falsified (West 1960). Akin (1966) suggested the involvement of an unknown self-inhibiting agent. Other works related this inhibition to the production of some "proteinaceous" compounds by large tadpoles (Rose and Rose 1961, Runkova et al. 1974, Stepanova 1974, Steinwascher 1978). Notably, Richards (1962) showed that growth inhibition could be removed after physical and chemical treatments like filtration, centrifugation, heating, sonication, freezing and thawing, ultraviolet light and low pH. We propose that all these observations can coherently be ascribed to the species-specific inhibitory effects of exDNA accumulated in the growth medium.

A distinct topic where the specificity of action of exDNA could play an important role is self-recognition. Callaway and Mahall (2007) reviewed the evidence regarding how plants are able to distinguish self from non-self conspecific individuals. In particular, Dudley and File (2007) demonstrated kin recognition at root level in *Cakile edentula* without proposing an explanatory mechanism. Considering the high specificity of the information stored in DNA, we speculate that it can potentially mediate recognition not only at species level, but also within species to distinguish kin from unrelated individuals.

In this work, we presented phenomenological evidence supporting the hypothesis of the general occurrence of an inhibitory effect of extracellular self-DNA and of its possible involvement in recognition signalling processes. Are these functions of exDNA going to be a new paradigm? The reported findings certainly suggest intriguing questions and ideas, which may open new research scenarios. For example, in ecology, experiments can be planned to investigate the relevance of this effect in the regulation of species coexistence and competition, in the interactions with natural enemies, in relation with nutrient depletion and symbiont community changes, and its general occurrence in natural conditions. Moreover, a more comprehensive experimental design should address the relationship between inhibition and phylogenetic distance among target species and exDNA sources.

In a broader context of life sciences, other issues can be considered. The reported species-specificity of DNA inhibition seems consistent in eukaryotes (both unicellular and multicellular organisms), but this should be further investigated on a larger number of taxa. On the other hand, the effect on prokaryotes appears less certain considering that heterologous DNA also produced a performance reduction in the only observed case of *Bacillus subtilis*. This definitely requires further experimental work on more species.

Finally, the investigation of the molecular mechanisms behind the observed inhibitory phenomenon is certainly a major challenge to be faced. It has been widely demonstrated that exDNA can be uptaken by living cells in both prokaryotes and eukaryotes, such as higher plants (Paungfoo-Lonhienne et al. 2010) and mammalian (Groneberg et al. 1975) where it can be transported to the nucleus (Wienhues et al. 1987) and possibly integrated into the genome of the guest cell (Doerfler et al. 1995). Indeed, cells present mechanisms of protection from exDNA uptake. Bacterial restriction enzymes cleave foreign nucleic acids while protecting their own genome by methylation (Wilson 1988). More sophisticated processes of specific clearance of exDNA are found in vertebrates (e.g. Stenglein 2009). The above mentioned mechanisms refer to the recognition of exogenous DNA, whereas little is known about the processes involved in specific responses to self-DNA, for which the mechanisms of viral, retroviral transposons, or other types of parasitic DNA could be taken into account. Future studies are needed to clarify the inhibitory effects of extracellular self-DNA at both cellular and molecular levels, including the processes of recognition, uptake, and transport in both prokaryotes and eukaryotes.

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Tables

Table 1. Summary of the general linear mixed model (GLMM) testing for main and interactive effects of target species and treatments on species performance in the bioassays.

a) Model I: self and heterologous DNA						
	Effect type	SS	df	MS	*F*	*P*
Target species	Random	2134.7	5	426.9	1.53	0.2656
Treatment	Fixed	88928.9	2	44464.4	159.60	< 0.0001
Target species x Treatment	Random	2822.9	10	282.3	7.66	< 0.0001
b) Model II: concentration of self-DNA						
	Effect type	SS	df	MS	*F*	*P*
Target species	Random	18277.5	5	3655.5	6.55	0.0077
Concentration	Fixed	21909.3	2	10954.7	20.13	0.0005
Target species x Concentration	Random	5095.7	9	566.2	14.91	< 0.0001

Table 2. Performance of target species exposed to extracellular heterologous DNA from *Arabidopsis thaliana* and self-DNA at different concentration levels. Data are mean ± standard deviations of different growth metrics for different species, expressed as % of untreated controls. Within each target species, asterisks indicate significant difference between exposure to heterologous and self-DNA at high concentration (Duncan post-hoc tests for the effect of treatment from GLMM model I in Table 1). Different letters indicates significantly different groups for the effect of self-DNA concentration (Duncan post-hoc tests from GLMM model II in Table 1). Values not significantly different from the controls are reported in italic fonts.

Target species	H DNA	self-DNA		
	high	high	mid	low
Bacillus subtilis	58.2 ± 7.4 *	7.7 ± 5.6 *a*	6.0 ± 2.6 *a*	41.4 ± 6.5 *b*
Physarum polycephalum	*93.9 ± 7.5* *	0.7 ± 0.2 *a*	18.4 ± 3.9 *b*	44.7 ± 7.5 *c*
Scenedesmus obliquus	*95.8 ± 6.7* *	14.1 ± 6.7 *a*	-	60.6 ± 3.4 *b*
Trichoderma harzianum	*93.3 ± 9.0* *	9.1 ± 3.0 *a*	53.0 ± 10.0 *b*	67.0 ± 16.0 *c*
Acanthus mollis	*94.8 ± 8.7* *	26.8 ± 1.4 *a*	81.7 ± 3.7 *b*	*98.1 ± 5.4 c*
Sarcophaga carnaria	*96.1 ± 4.0* *	12.5 ± 4.0 *a*	11.7 ± 3.0 *a*	44.2 ± 8.0 *b*

Table 3. Performance of target species exposed to extracellular heterologous DNA from different sources. Data are mean ± standard deviations of different growth metrics for different species, expressed as % of untreated controls. Values not significantly different from the controls are reported in italic fonts.

Target species	Source of heterologous DNA				
	Escherichia coli	*Bacillus subtilis*	*Aspergillus niger*	*Sarcophaga carnaria*	*Quercus ilex*
Bacillus subtilis	51±13%	-	62±24%,	42±13%	-
Trichoderma hartianum	-	*108±14%*	*91±11%*	*98±9%*	-
Acanthus mollis	-	-	-	*102±11%*	*94±19%*

Figure Legends

Figure 1. Effects of exposure to heterologous DNA from *Arabidopsis thaliana* and self-DNA on different organisms. All species show significant concentration dependent inhibitory effects by self-DNA. See Materials and Methods for details on experimental conditions.

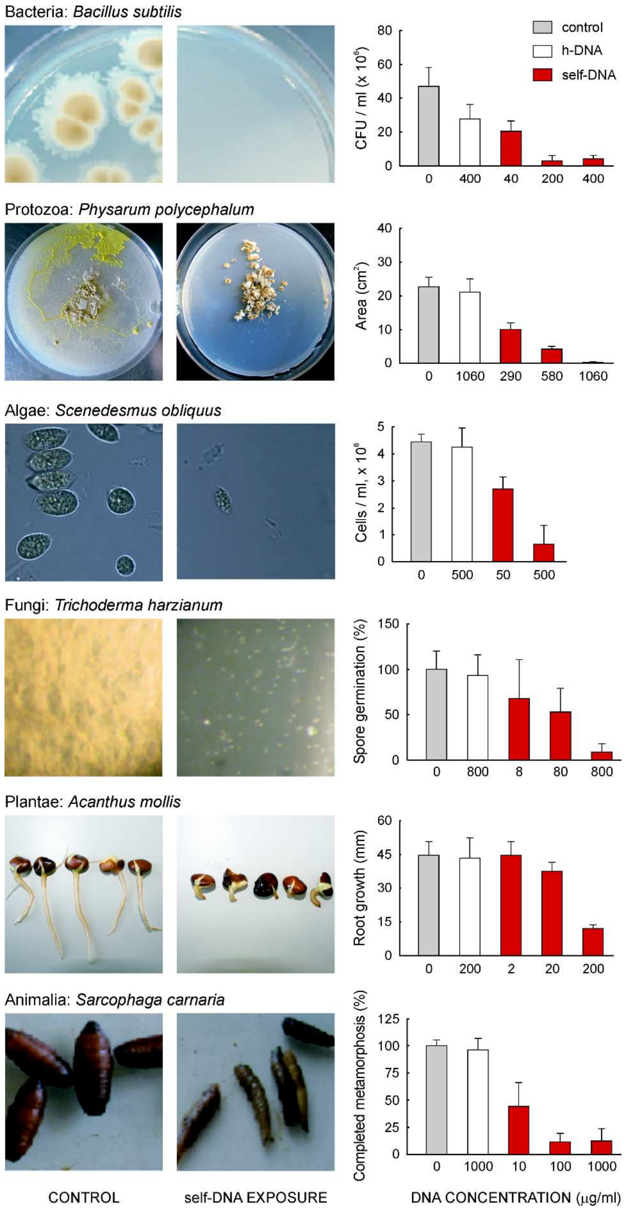
Bacteria: Bacillus subtilis
CFU / ml (x 10^6)
control
h-DNA
self-DNA
0 400 40 200 400
Protozoa: Physarum polycephalum
Area (cm²)
0 1060 290 580 1060
Algae: Scenedesmus obliquus
Cells / ml, x 10^6
0 500 50 500
Fungi: Trichoderma harzianum
Spore germination (%)
0 800 8 80 800
Plantae: Acanthus mollis
Root growth (mm)
0 200 2 20 200
Animalia: Sarcophaga carnaria
Completed metamorphosis (%)
0 1000 10 100 1000
CONTROL
self-DNA EXPOSURE
DNA CONCENTRATION (μg/ml)

Figure 1