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Virulence test using nematodes to prescreen *Nocardia* species capable of inducing neurodegeneration and behavioral disorders

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Background. Parkinson's disease (PD) is a disorder characterized by dopaminergic neuron programmed cell death. The etiology of PD remains uncertain—some cases are due to selected genes associated with familial heredity, others are due to environmental exposure to toxic components, but over 90% of cases have a sporadic origin. *Nocardia* are Actinobacteria that can cause human diseases like nocardiosis. This illness can lead to lung infection or central nervous system (CNS) invasion in both immunocompromised and immunocompetent individuals. The main species involved in CNS are *N. farcinica*, *N. nova*, *N. brasiliensis* and *N. cyriacigeorgica*. Some studies have highlighted the ability of *N. cyriacigeorgica* to induce Parkinson's disease-like symptoms in animals. Actinobacteria are known to produce a large variety of secondary metabolites, some of which can be neurotoxic. We hypothesized that neurotoxic secondary metabolite production and the onset of PD-like symptoms in animals could be linked. **Methods.** Here we used a method to screen bacteria that could induce dopaminergic neurodegeneration before performing mouse experiments. **Results.** The nematode *Caenorhabditis elegans* allowed us to demonstrate that *Nocardia* strains belonging to *N. cyriacigeorgica* and *N. farcinica* species can induce dopaminergic neurodegeneration. Strains of interest involved with the nematodes in neurodegenerative disorders were then injected in mice. Infected mice had behavioral disorders that may be related to neuronal damage, thus confirming the ability of *Nocardia* strains to induce neurodegeneration. These behavioral disorders were induced by *N. cyriacigeorgica* species (*N. cyriacigeorgica* GUH-2 and *N. cyriacigeorgica* 44484) and *N. farcinica* 10152. **Discussion.** We conclude that *C. elegans* is a good model for detecting *Nocardia* strains involved in neurodegeneration. This model allowed us to detect bacteria

with high neurodegenerative effects and which should be studied in mice to characterize the induced behavioral disorders and bacterial dissemination.

1 **Virulence test using nematodes to prescreen *Nocardia* species**
2 **capable of inducing neurodegeneration and behavioral disorders**

3

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18

19 Short title: Dopaminergic neuron degradation by *Nocardia*

20

21 **Abstract**

22

23 **Background.** Parkinson's disease (PD) is a disorder characterized by dopaminergic neuron
24 programmed cell death. The etiology of PD remains uncertain—some cases are due to selected
25 genes associated with familial heredity, others are due to environmental exposure to toxic
26 components, but over 90% of cases have a sporadic origin. *Nocardia* are Actinobacteria that can
27 cause human diseases like nocardiosis. This illness can lead to lung infection or central nervous
28 system (CNS) invasion in both immunocompromised and immunocompetent individuals. The
29 main species involved in CNS are *N. farcinica*, *N. nova*, *N. brasiliensis* and *N. cyriacigeorgica*.
30 Some studies have highlighted the ability of *N. cyriacigeorgica* to induce Parkinson's disease-
31 like symptoms in animals. Actinobacteria are known to produce a large variety of secondary

32 metabolites, some of which can be neurotoxic. We hypothesized that neurotoxic secondary
33 metabolite production and the onset of PD-like symptoms in animals could be linked.

34 **Methods.** Here we used a method to screen bacteria that could induce dopaminergic
35 neurodegeneration before performing mouse experiments.

36 **Results.** The nematode *Caenorhabditis elegans* allowed us to demonstrate that *Nocardia* strains
37 belonging to *N. cyriacigeorgica* and *N. farcinica* species can induce dopaminergic
38 neurodegeneration. Strains of interest involved with the nematodes in neurodegenerative
39 disorders were then injected in mice. Infected mice had behavioral disorders that may be related
40 to neuronal damage, thus confirming the ability of *Nocardia* strains to induce neurodegeneration.
41 These behavioral disorders were induced by *N. cyriacigeorgica* species (*N. cyriacigeorgica*
42 GUH-2 and *N. cyriacigeorgica* 44484) and *N. farcinica* 10152.

43 **Discussion.** We conclude that *C. elegans* is a good model for detecting *Nocardia* strains
44 involved in neurodegeneration. This model allowed us to detect bacteria with high
45 neurodegenerative effects and which should be studied in mice to characterize the induced
46 behavioral disorders and bacterial dissemination.

47

48 Introduction

49 Parkinson's disease (PD) is the second most frequent neurodegenerative disorder after
50 Alzheimer's disease. With the rise in the population mean age, the prevalence of this illness is
51 increasing, affecting millions of individuals worldwide. PD is a slowly evolving disorder
52 characterized by bradykinesia, rigidity, tremor and postural instability. The pathological
53 hallmark of PD is the degeneration of dopaminergic neurons localized in the *substantia nigra*
54 *pars compacta*, resulting in loss of the nigrostriatal pathway and a reduction of dopamine levels
55 in the striatum (Braak et al., 2003). For many years, PD was considered a nongenetic disorder
56 caused by synergistic environmental factors. Large genome-wide association studies (GWAS)
57 have identified more than two dozen common genetic variants for PD, each with a relatively
58 small effect size; in combination with rare Mendelian genes, genetics account for at best 10–20
59 % of PD (Lil et al., 2012; Nalls et al., 2014; Trinh and Farrer, 2013). The majority of PD cases
60 have a sporadic origin, and the environment seems to have a critical impact on the epidemiology
61 of this illness (Goldman, 2014; Ritz et al., 2016). Several studies have suggested that rural
62 environments may be epidemiological contributors to PD. It is well known that pesticides and

63 herbicides like rotenone, paraquat, and MPTP are etiologic agents of PD (Hatcher et al., 2008;
64 Khandhar & Marks, 2007). Indeed, these molecules are lipophilic, they are able to cross the
65 blood-brain barrier, the neuronal cellular membrane and cause oxidative stress, in turn inducing
66 neurodegeneration. Animal models of PD involving these pesticides have been developed by
67 several research teams. The action of these toxins was noted, and a dysfunction in the ubiquitin-
68 proteasome system (UPS) involved in protein degradation has also been frequently observed.
69 Toxins that can inhibit the UPS have been identified as secondary metabolites produced by
70 microorganisms. For instance, proteasome inhibitors like epoxomicin and lactacystin can cause
71 impairment of the UPS responsible for neurodegeneration in animal models (McNaught et al.,
72 2004; Salama & Arias- Carrión, 2011).

73 *Nocardia* are aerobic Gram-positive actinomycetes bacteria with a high G+C percentage. They
74 are important components of the soil microflora and can also be found in fresh and salt water
75 environments (Brown-Elliot et al., 2006; Wilson, 2011). Until now, more than 80 *Nocardia*
76 species have been described in the literature, with 33 being responsible for human diseases
77 (Abreu et al., 2015; Brown-Elliot et al., 2006; Wilson, 2011). These bacteria can be aerosolized
78 in dust, which can be inhaled (Ambrosioni, Lew & Garbino, 2010; Brown-Elliot et al., 2006) and
79 lead to lung infections. The central nervous system is the second most commonly infected organ
80 by *Nocardia* spp. (Beaman et al., 1976; Ogata & Beaman, 1992). In humans, cerebral nocardiosis
81 may cause the following symptoms: nausea, vomiting with photophobia, headache, neck
82 stiffness, motor disorders (hemiparesis and tremors) and behavioral disorders (schizophrenia,
83 depression, dyslexia, hallucinations and amnesia) (Beaman & Beaman, 1994). Khobata &
84 Beaman, 1991 reported that a sublethal injection of *Nocardia cyriacigeorgica* GUH-2 can induce
85 a syndrome in mice which shares clinical and pathological similarities with PD. These results
86 were confirmed in other studies (Khobata & Beaman, 1991; Ogata & Beaman, 1992).

87 *Streptomyces venezuale*, another actinomycete, was also described as being able to produce
88 secondary metabolites which could induce dopaminergic neurodegeneration (Caldwell et al.,
89 2009).

90 The aim of this study was to develop a method to study *Nocardia* properties involved in neuronal
91 virulence and assess the health risks that various *Nocardia* species isolated from clinical and
92 environmental samples may represent. This test was designed so that the number of isolates
93 analyzed would be higher than in the mouse model. This method consists of performing a test on

94 the nematode *C. elegans* that was previously described as a good model for studying
95 neurotoxicity induced by *S. venezuelae* (Caldwell et al., 2009; Harrington et al., 2010, Martinez
96 et al., 2017). *C. elegans* has 302 neurons, eight of which are dopaminergic neurons. These
97 dopaminergic neurons are located in the nematode as follows: (i) six are in the anterior part of
98 the nematode and consist of two pairs of cephalic neurons (CEP) and one pair of class *E* anterior
99 deirid neurons (ADE), and (ii) two class *E* posterior deirid neurons (PDE) in the posterior part of
100 the animal (Fig. 1) (Berkowitz et al., 2008; Locke et al., 2008). Modifications in these structures
101 may indicate a neurotoxic effect of the bacterial supernatant.

102

103

104 **Materials and methods**

105

106 ***Nocardia* strains**

107 Table 1 indicates the *Nocardia* strains used in this study. *Nocardia* strains were grown at 37°C
108 shaking at 150 rpm in BHI-P medium (for BALB/c mouse experiments) and in Bennett liquid
109 medium (for nematode tests) because BHI-P medium was toxic for nematodes. Then, for tests
110 on nematodes, culture supernatants were recovered after a 1 month incubation period for *N.*
111 *farcinica* IFM 10152 and 2 months for *N. cyriacigeorgica* and *N. asteroides* strains. These
112 conditions were defined according results obtained in preliminary tests. For the BALB/c mouse
113 tests, *Nocardia* cells were grown in order to recover 3,5. 10⁵ CFU.mL⁻¹.

114

115 **Nematode neurodegeneration assay**

116 The *C. elegans* BY250 *vtIs7* [Pdat-1:GFP]) line was used. This is a transgenic line specifically
117 expressing GFP in dopaminergic neurons (*dat-1* promotor) (Khobata & Shimokawa, 1993). The
118 integrity of the six anterior dopaminergic neurons was monitored with this *C. elegans* line. In our
119 experiments, *C. elegans* strains were cultured on NGM medium and fed with *E. coli* OP50 at
120 23°C according to standard methods (Brenner, 1974; Hope, 1999). Gravid nematodes were
121 dropped onto plates and removed around 6 h later, leaving time for egg laying. Eggs were then
122 incubated for 3 days at 15°C. Nematodes at the L4 development stage were then picked up and
123 dropped onto NGM medium supplemented with 10 µM 5-FU (5-fluorouracil). The same

124 experiment was done without 5-fluorouracil and we obtained different nematode development
125 stages. This variability had an effect on their neuronal viability, probably due to their age. 5-FU
126 was thus used to block the development of new eggs in order to standardize the assay. This step
127 represented day 0 of the experiment. Nematodes were plated with filtered Bennett supernatants
128 recovered from *Nocardia* broth. Supernatants were recovered from the first plating of nematodes
129 (egg-laying period) and then at days 0, 2 and 4 at 23°C. Some nematodes were exposed to
130 sterile Bennett broth for control. At day 6, for each bacterial supernatant tested, 30 nematodes
131 per condition were placed on 2% agarose pads, fixed with tetramisol (5 mM) and observed by
132 fluorescence microscopy with a GFP38He filter. Microscopic analyses were performed with an
133 Axio imager.Z1 (Zeiss). Nematodes were considered as having a wild-type phenotype when they
134 showed no neuronal abnormalities. Nematodes with dendrite blebbing or beading, neuronal cell
135 body rounding, or cell body and/or process loss were considered as affected. Blebbing and
136 beading are different modifications along the axonal process. Blebbing can be defined as
137 triangular-shaped protrusions, and beading as focal enlargements, but here we do not
138 differentiate these two terms, and use the generic term “blebbing” for both phenomena (Chew et
139 al., 2013). Behavioral tests for dopamine function were performed using: (i) a touching test on
140 nematodes, and (ii) body-bend counting (one body bend is deemed as one sinusoidal movement
141 until the worm reaches the same posture again). The first test consists of touching the nose of the
142 nematodes and in observing their behavioral reactions. The second involves counting body-bends
143 per minute for 20 nematodes per condition (Taferner et al., 2015; Yu and Liao, 2014). The wild-
144 type *C. elegans* line (N2) and a transgenic line (BY250) was used for this behavior test.

145

146 **BALB/c experiments**

147 Female BALB/c mice weighing 18 to 20 g were used, and handled in a level 2 safety lab at
148 Claude Bourgelat Institute ® (Vetagro Sup, Marcy l’Etoile, France). ISOcages™ were used for
149 this experiment. Animals were acclimated for 10 days to their environment prior to testing. All
150 experiments were approved by the VetAgro Sup ethics committee (authorization number 722).
151 Each BALB/c mouse received a sublethal injection of *Nocardia* (around $3,5 \cdot 10^5$ CFU.mL⁻¹)
152 through the lateral tail vein, as described by Kohbata and Beaman, 1991 (Kohbata & Beaman,
153 1991). Behavioral disorders in mice were observed 13 days after infection. The behavioral
154 disorders were: hemiparesis, muscular rigidity, tremors throughout the body or vertical head

155 movements. Mice selected for anatomo-pathology analyses were those having the most severe
156 symptoms. BALB/c mice were euthanized at the end of the experiments, after anesthesia
157 (intraperitoneal injection of ketamine (100 mg.kg⁻¹)), by an intraperitoneal injection of 0.5ml of a
158 dolethal solution. Some organs were collected. Brains were cut to separate the two hemispheres.
159 The first part was frozen in liquid nitrogen and conserved at -80°C, the second part was
160 immersed in histological buffered formalin (pH 7.4-7.6), for further analyses. After fixation in
161 histological buffered formalin, organs were dehydrated using five successive ethanol baths (first
162 70°, second 90° and third close to absolute ethanol) and then were introduced in three butyl
163 ethanol baths. Finally, samples were immersed in a paraffin bath at 60°C. Serial sections 4 µm
164 thick were cut from the paraffin organ blocks. Each series of six cups were done every 400 to
165 500 µm to be representative of the entire organ. Each series was stained differently: Harris-eosin
166 hematoxylin stain, Fite stain, Gram stain, histochemical and immunochemical stain. Rabbit anti-
167 mycobacterium polyclonal antibody (SEROTEC OBT0947) was used for histochemical and
168 immunochemical staining.

169

170 **Statistical tests**

171 Statistical tests were performed with the R v.2.14.0 package (<http://www.r-project.org/>). Fisher
172 exact tests were performed between strains and controls in the nematode experiments
173 (acceptance threshold 5%). For the mouse experiments, we conducted this test between
174 treatments and the number of mouse deaths or between strains and controls. For tests on
175 nematodes, the experiment was repeated five times for *N. cyriacigeorgica* GUH-2 and *N.*
176 *farcinica* 10152 to validate the test. The other strains (Table 1) were tested twice or three times
177 each.

178

179 **Results**

180 **Bacterial induction of dopaminergic neurodegeneration**

181 The neurotoxicity of metabolites excreted by *N. cyriacigeorgica*, *N. asteroïdes* and *N. farcinica*
182 (Bennett medium culture) was tested on the nematode *C. elegans* targeted with GFP on
183 dopaminergic neurons receptors. When the nematodes were exposed to bacterial supernatant for
184 10 days, dendrite blebbing, neuronal cell body rounding, or cell body and/or process loss were

185 monitored. Deformed neurons and blebbing processes were repeatedly monitored, but neuronal
186 loss seldom occurred (Fig. 2). Significant effects on the degeneration of *C. elegans* dopaminergic
187 neurons ($p < 0.05$) were observed for *N. cyriacigeorgica* GUH-2, *N. cyriacigeorgica* N27, *N.*
188 *cyriacigeorgica* 04.100 and *N. farcinica* IFM 10152 culture supernatants (Table 2). For *N.*
189 *cyriacigeorgica* GUH-2, 36.7% (11/30) nematodes were affected: 82% showed dendrite
190 blebbing, 73% neuronal cell body rounding and 9% neuronal loss. For *N. cyriacigeorgica* N27,
191 33.3% (10/30) nematodes were affected and, among them, 90% had dendrite blebbing and 50%
192 showed neuronal cell rounding. For *N. farcinica* IFM 10152, 53% (17/32) nematodes were
193 affected: 70.5% of these showed dendrite blebbing, 70.5% neuronal cell rounding, and 23.5%
194 neuronal cell loss (Table 2). Fisher exact tests indicated that the findings for two strains were
195 close to significance: *N. cyriacigeorgica* 04.100, with 30% (9/30) of nematodes affected and *N.*
196 *asteroides* ATCC19247 with 25.8% (8/31) of nematodes affected. Taking the overall
197 populations into account, we could not draw clear conclusions for both strains, but the marked
198 difference in the significance levels obtained for these two strains indicated that *N.*
199 *cyriacigeorgica* 04.100 had an effect on neurons ($p = 0.042$), while *N. asteroides* ATCC19247
200 had no effect ($p = 0.082$). We also performed a behavioral test for the dopamine function using a
201 nematode touch sensitivity test; firstly to ensure that the nematodes were still alive and,
202 secondly, to detect dopamine function alterations. *N. farcinica* 10152 or *N. cyriacigeorgica*
203 GUH-2 strains induced higher neurodegeneration (Table 2) and, for these strains, we observed
204 nematode behavioral disorders. The control nematodes (N2 and BY250) had functional
205 dopaminergic neurons and the touch responses included receding movement followed by rapid
206 forward leak. When nematodes were in contact with supernatant from *N. farcinica* 10152 or *N.*
207 *cyriacigeorgica* GUH-2, we noted the same behaviours as those observed without supernatants,
208 but the nematode movements were very slow or only backwards. We also observed new
209 behaviours: saccadic forward and backward movements without forward leak or motionless
210 nematodes with only nose movements (Table S1). We performed a second test to quantify the
211 behavioral phenotypes for dopaminergic functions. This test consisted of counting nematode
212 body-bends per minute (Liu et al., 2015). For the controls (N2 and BY250) without supernatant,
213 we counted 12 body-bends/min for N2 (WT strain) and 14.1 body-bends/min for BY250
214 (transgenic worms with GFP expression). Regardless of the nematode strain tested, worms had
215 decreased mobility with all supernatants tested (4.5 and 9.4 body-bends/min for *N. farcinica*

216 10152 and *N. cyriacigeorgica* GUH-2 with *C. elegans* N2 and 5 and 9.75 body-bends/min for *N.*
217 *farcinica* 10152 and *N. cyriacigeorgica* GUH-2 with *C. elegans* BY250). For both nematode
218 lines, the supernatants had significant effects (Fig. 3).

219

220 **Mouse behavioral disorders induced by *Nocardia***

221 Mice were infected with a sublethal bacterial suspension (Beaman & Beaman, 1994). Three
222 *Nocardia* species were tested, i.e. two clinical strains of *N. cyriacigeorgica*, one clinical strain of
223 *N. farcinica*, and one environmental strain of *N. asteroides* (Table 1). The non-virulent status of
224 *N. asteroides* 19247 defined by Beaman 1996, Beaman and Beaman 1998 (Beaman 1996,
225 Beaman & Beaman 1998) was confirmed in this study (Table 3). The other strains induced
226 behavioral disorders from day 6 post-infection (Table 3). Indeed, the number of mice with such
227 disorders (and their intensity) increased until day 13 post-infection. These disorders were due to
228 muscular rigidity and hemiparesis (supplementary material video link). The latter disorder was
229 essentially visible by the position of the head, which was falling on one side. These damaged
230 mice tended to turn in the same direction and begin to turn quickly when they were held by the
231 tail. We also observed whole body tremors in some mice. Rhythmical and vertical movements of
232 the head were also observed (supplementary material video link). These movements occurred
233 more than 50 times in 30 s (Table S1), they were very characteristic and different from control
234 mouse movements. Mice infection with *N. farcinica* 10152 had more severe symptoms than
235 those infected with *N. cyriacigeorgica*. Indeed, 45% of the mice (9/20) showed behavioral
236 disorders after injection. A lethal dose (around 10^7 CFU) of *N. cyriacigeorgica* GUH-2 was
237 tested, which led to 50% mortality within 5 days post-injection.

238

239 **Histology**

240 Necropsies for organ histological analysis were performed on mice that received a lethal
241 injection of *N. cyriacigeorgica* GUH-2. Macroscopic observations revealed the presence of soft
242 beige nodules on the spleen, kidney, myocardium, brain, liver and lung tissues. The organ
243 histological findings revealed the presence of infectious foci. The largest lesions affected the
244 kidney, spleen and myocardium. Lesions were characterized by abscesses, larger concentrations
245 of inflammatory cells (poly- and mono-nuclear) and diffuse infiltration of these cells in the
246 interstitial tissues. The kidney histological findings revealed the presence of filamentous bacteria

247 strongly evocative of *Nocardia* (Fig. 4). These observations confirmed the dissemination of
248 *Nocardia* throughout the body.
249 Brains of mice that had received a sub-lethal injection of *N. cyriacigeorgica* GUH-2 were
250 recovered and analyzed. Analysis of sagittal brain slices revealed the presence of lesions of the
251 gliosis cluster located at the bottom middle part of the telencephalon. An encephalon of a mouse
252 infected with *N. cyriacigeorgica* GUH-2 but without motor symptoms revealed the presence of
253 little gliosis clusters at the base of telencephalon with Harris-eosin hematoxylin staining (data
254 not shown). There was slight inflammation at the base of cerebral hemispheres, but *Nocardia*
255 was not revealed by staining (Fite, Gram, histochemical and immunochemical staining).
256 Observations on a brain recovered from a mouse presenting with motor symptoms (infected by
257 strain *N. cyriacigeorgica* 44484) showed the presence of a diffuse gliosis at the base of the
258 telencephalon and a small perivascular lymphocytic sleeve in the medulla oblongata. A little
259 gliosis cluster was seen at the base of the telencephalon and one hyperchromatosis of neurons in
260 the medulla oblongata (data not shown). The brains of mice with behavioral disorders (infected
261 by *N. farcinica* 10152) showed a gliosis cluster at the base of the telencephalon, with Harris-
262 eosin hematoxylin staining (Fig. 5A). The encephalon of one mouse showing hemiparesis, after
263 infection with *N. farcinica* 10152 showed, by Harris-eosin hematoxylin staining, three gliosis
264 clusters, one on the diencephalon and two at the base of the telencephalon, (Fig. 5B). Fite
265 staining revealed the presence of *Nocardia*-like cells (Fig. 5C). Histochemical and
266 immunochemical staining highlighted *Nocardia*-like cells in the cerebellum and in the *medullae*
267 *oblongatae* (Fig. 5D). It is noteworthy that at five weeks post-inoculation, *Nocardia*-like cells
268 were only observed in mice with hemiparesis.

269

270 Discussion

271 *Nocardia* strains were found to induce behavioral changes in mice, and some of their excreted
272 metabolites could cause neuronal degeneration in the nematode *C. elegans*. Our data suggests
273 that the transgenic strain BY250 *vtIs7* [Pdat-1:GFP] could be useful for investigating chemically-
274 induced neurodegeneration. This nematode line allowed the detection of *Nocardia* strains
275 producing secondary metabolite(s) in the broth, which may induce brain damage. This led to the
276 first observation of a *N. farcinica* strain inducing behavioral disorders in mice. These results

277 indicate that the ability to induce neurodegeneration could be widely distributed in the *Nocardia*
278 genus.

279

280 **Dopaminergic neuron neurodegeneration**

281 The *N. cyriacigeorgica* GUH-2 strain can invade the neuronal central system and cause
282 dopaminergic neurodegeneration in mice (Ogata & Beaman, 1992). Here we demonstrate that
283 this property induced by *N. cyriacigeorgica* can be obtained using a rapid test with the *C.*
284 *elegans* BY250 *vtIs7* [Pdat-1:GFP]) line. This test, that involved exposing *Nocardia* supernatants
285 to nematodes, highlighted damage on dopaminergic neurons. Supernatants were used because we
286 hypothesized that dopaminergic neurodegeneration was due to metabolic compounds secreted by
287 these bacteria. Thus, nematodes exposed to supernatants allowed us to test for the presence of
288 metabolites involved in bacterial virulence. It is well known that pathogenesis may be connected
289 to excreted metabolites among *Actinobacteria*. For example, nocobactine, a siderophore, was
290 found to contribute to the cytotoxicity of *N. farcinica* 10152 (Hoshino et al., 2011; Ishikawa et
291 al., 2004). The same was noted with mycobactin, a *M. tuberculosis* siderophore (Krithika et al.,
292 2006). These two siderophores are products of secondary metabolism. *Nocardia* is known to
293 produce some of these virulence factors. For example, *N. cyriacigeorgica* GUH-2 supernatants
294 have apoptotic activity on PC12 culture cells with inhibition of the three enzymatic activities of
295 PC12 proteasomes and inhibition of only two of them for human proteasomes (Barry & Beaman,
296 2007). The major interest of this nematode test is the possibility of screening a large number of
297 bacterial strains for their neurodegenerative potential before, or instead, of using mammalian
298 models. In this study, seven *Nocardia* strains of environmental and clinical origin were tested.
299 The results showed the ability of four *N. cyriacigeorgica* strains to significantly damage the
300 neuronal system, including *N. cyriacigeorgica* 44484, which induced neuronal body loss but not
301 significantly. This was probably due to a low number of observed nematodes. The statistical
302 analysis findings would likely be stronger if we had increased the number of worms tested. This
303 property did not seem to be restricted to the *N. cyriacigeorgica* GUH-2 strain as we had
304 previously thought. In fact, the *N. cyriacigeorgica* N27 strain produced secondary metabolites
305 that could substantially damage dopaminergic neurons. *N. farcinica* IFM 10152 had the same
306 effect on nematodes. These excreted metabolites involved in virulence were detected in broths
307 from clinical (i.e. GUH2, IFM 10152, 04.100) and environmental strains (i.e. N27). Human

308 exposure to virulent *Nocardia* mainly occurs through contact with environmental matrices where
309 this bacterium is present. This test thus confirmed the health hazards associated with
310 environmental strains. However, the distribution of such metabolites involved in virulence
311 among the various *Nocardia* species remains to be explored. Supernatants of non-virulent strains
312 did not lead to neuronal degeneracy.

313 The *N. cyriacigeorgica* N27 strain was isolated from a hydrocarbon-contaminated environment
314 (results not shown). Environmental exposure to such a pathogen is possible for populations in
315 contact with highly hydrocarbon contaminated environments such as urban areas. More
316 environmental *Nocardia* species could likely induce the same symptoms and this needs to be
317 further explored. This test will be applied to assess a larger panel of species and strains. Neuronal
318 damage induction is not exclusive to *Nocardia* and can be found in other bacterial genera such as
319 *Streptomyces* (Caldwell et al., 2009). Caldwell *et al.* (2009) showed that *S. venezuelae* could
320 induce effects neurons similar to those observed with *Nocardia* secreted metabolites. After
321 testing the potential of different *Streptomyces* strains to induce dopaminergic neuron
322 degeneration in *C. elegans*, *S. venezuelae* was found to have a significant effect on nematodes
323 after four days of exposure to the culture supernatant. Nematodes in contact with supernatants
324 had damaged neurons that were deformed and showed blebbings, as also noted in our study
325 (Caldwell et al., 2009). It is well known that blebbing frequency appearance can increase with
326 age of nematode but these aged neurons are not undergoing apoptosis or necrosis (Chew et al.,
327 2013). All experiments were carried out in comparison with controls (Table 2). Only one or two
328 nematodes had neuronal structure modifications out the 30 nematodes tested. These neuronal
329 anomalies were likely due to the nematode age, for the other ones we did not have issues with
330 the nematode age. We took account of the controls in our statistical analyses (Table 2). We
331 considered the possible decrease in fluorescence when using GFP. However, if our results had
332 been partially due to a decrease in GFP expression, we would have also observed a loss of
333 fluorescence along the axon. In our experiments, as we retained fluorescence along the axon for
334 the controls and tests, we conclude that the results were not due to decreased of GFP expression.
335 These results were confirmed by the findings of the two behavioral tests performed and the use
336 of wild-type and transgenic nematode strains. We observed modifications in nematode behavior
337 related to dopaminergic neurons, like movements induced by a touch sensitivity test and the
338 mobility (body-bends) of the worms. We obtained the same results with both nematode strains

339 (N2 and BY250), so we conclude that the observed effect was due to the bacterial supernatant. In
340 our experiment, all nematodes were of the same age because we selected nematodes at the L4
341 development stage, so the differences observed between strains must have been due to the
342 secreted metabolites. Regarding the number of nematodes affected and the severity of the
343 induced disorders, metabolites from *N. farcinica* 10152 had stronger neurotoxic effects than *N.*
344 *cyriacigeorgica* GUH-2. In further analyses, a nematode with other neuronal GFP markers will
345 be used to see if our results are specific to dopaminergic neurons or if metabolites secreted by
346 *Nocardia* strains could affect other kinds of neurons.

347

348

349 **Behavioral disorders in mice and histology of encephala**

350 *Nocardia* species which induced neurodegeneration in nematodes (including strains inducing
351 neuronal body process loss) were tested in mice to confirm the onset of behavioral disorders in
352 the mammalian model. The results obtained showed the implication of *Nocardia* strains in the
353 onset of behavioral disorders in mice. Analyses of brain slices revealed lesions at the base of the
354 telencephalon likely responsible for the observed responses in mice. These observations were
355 generally in line with those of Kohbata and Beaman., 1991. All strains tested led to significant
356 difficulties for the mice to move forward, as shown in Beaman and Tam; 2008, but they did not
357 result in a vertical positioning of the tail (Kohbata & Shimokawa, 1993).

358 The histology of encephala showed the immune response of the infection (gliosis, lymphocytes)
359 but did not reveal the presence of *Nocardia* cells in mice with rhythmical and vertical
360 movements of the head, as observed by Pr Beaman (Beaman & Tam , 2008; Kohbata & Beaman,
361 1991) (Fig.5). *Nocardia* cells were observed in neurons of mice that had undergone hemiparesis
362 but also in kidney cells of mice that died of septicemia. These results revealed that new *Nocardia*
363 strains could be responsible for mouse behavioral disorders (*N. farcinica* 10152 and *N.*
364 *cyriacigeorgica* 44484). This is the first time that *N. farcinica* was shown to be involved in
365 movement disorders and detected among mouse brain tissues. Sequencing of the *N. farcinica*
366 IFM10152 genome revealed the presence of virulence genes, such as Mce proteins (mammalian
367 cell entry protein), antigen 85 family proteins, superoxide dismutase and factors involved in
368 adhesion and invasion of host cells, as noted in the *N. cyriacigeorgica* GUH-2 genome. These

369 proteins could be involved in the ability of *N. farcinica* to induce neuronal degeneration and this
370 hypothesis needs to be further explored (Ishikawa et al., 2004).
371 The mouse experiment results confirmed those obtained with nematodes. They confirmed that *N.*
372 *farcinica* 10152 was more virulent than *N. cyriacigeorgica* GUH-2 according to the severity of
373 the disorders observed. *N. cyriacigeorgica* 44484 induced neurodegeneration in mouse
374 experiments, but not significantly in nematode tests, even though we showed one neuronal body
375 process loss. This difference could be related to a lower level of production of the secondary
376 metabolites involved in the neurodegeneration of dopaminergic neurons with this strain. The
377 different culture time for *Nocardia* obtained with preliminary tests confirmed that *Nocardia*
378 species produce neurotoxic compounds at different rates. The results obtained with *N.*
379 *cyriacigeorgica* 44484 were important because they showed that *C. elegans* could be used in pre-
380 screening tests before performing mouse experiments, provided that neuronal body process loss
381 is taken into account. This difference between results in mice and nematode with this strain
382 indicates the need to take into account the growth rate precisely and the ODs which are
383 parameters difficult to control in *Actinobacteria*.

384

385 **Conclusion**

386 The aim of this study was to develop a method to investigate *Nocardia* properties involved in
387 neuronal virulence and assess the health hazards of *Nocardia* strains. We thus used the *C.*
388 *elegans* BY250 *vtIs7* [Pdat-1:GFP] line as a model system, and it seems to be a relevant model
389 for studying neuronal dopaminergic damage, as suggested previously (Ali & Rajini, 2012;
390 Harrington et al., 2010; Vistbakka et al., 2012).

391 In mice, we tested strains affecting dopaminergic neurons of nematodes, including those
392 inducing neuronal body process losses. This experiment revealed the ability of the bacteria to
393 induce behavioral disorders in the host animal while affecting neurological areas. Our results
394 confirmed those obtained by Kohbata & Beaman, 1991 and Beaman & Tam, 2008.

395 Our study revealed that *N. cyriacigeorgica* (not only the GUH-2 strain) and *N. farcinica* could
396 induce dopaminergic neuron degeneration in *C. elegans* and mice, despite their origins. In the
397 light of our results, *N. farcinica* 10152 seems to have had a greater neurotoxic effect on
398 dopaminergic neurons than other tested strains. Tests on the *C. elegans* BY250 *vtIs7* [Pdat-
399 1:GFP] line appeared to be faster and easier to perform than the mouse experiments for

400 detecting neurodegeneration, and this is a good model to screen numerous bacteria. This
401 nematode test could be a good model for bioactivity guided research on bioactive bacterial
402 compounds to find the molecule(s) responsible for dopaminergic neurodegeneration. We are
403 currently conducting some bioactivity guided research on active bacterial compounds. Active
404 fractions were obtained but chemical analyses showed that these fractions were too complex and
405 needed further purification to obtain purified active metabolites.

406

407

408

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414

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Figure 1

Dopaminergic neuron locations in *C. elegans* according to the WormAtlas

The neuronal body and the axons are shown in red. The green arrows indicate the four CEP neurons, the blue ones indicate the two ADE neurons and the orange ones indicate the PDE neurons. Only one PDE neuron is represented because the other one was behind the organs.

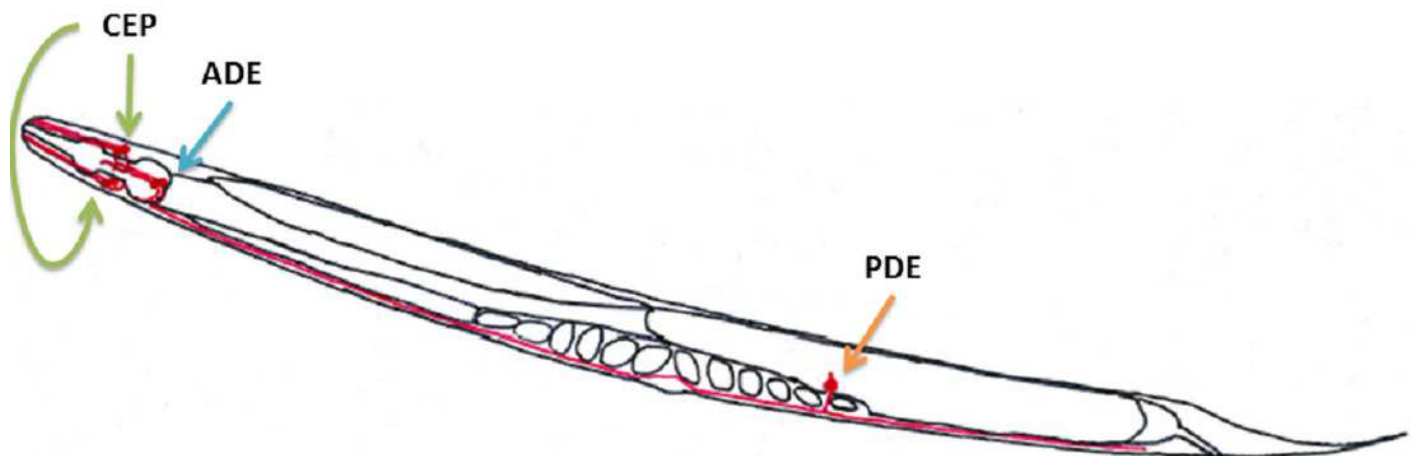


Figure 2

Fluorescent microscopy observation of *C.elegans* dopaminergic neurons

In (A), Head of *C. elegans* exposed to control supernatant with unaltered neurons. Yellow arrows indicate the four CEP neurons and the blue ones indicate the two ADE neurons. In (B), damaged head of *C. elegans*. The red arrows show four neurons (2 ADE and 2 CEP) still present and the axons had blebbing. Two CEP neurons showed no visible fluorescence. Nematodes exposed to *N. cyriacigeorgica* supernatant were used for this picture. In (C), the dendrites of dopaminergic neuron posterior (PDE) *C. elegans* exposed to control supernatant. In (D), dendrites of posterior dopaminergic neurons (PDE) with blebbing characterized by the appearance of visible dots along the axon. Nematode exposed to *N. farcinica* supernatant was used for this picture. Worms were observed through a X20 lens.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

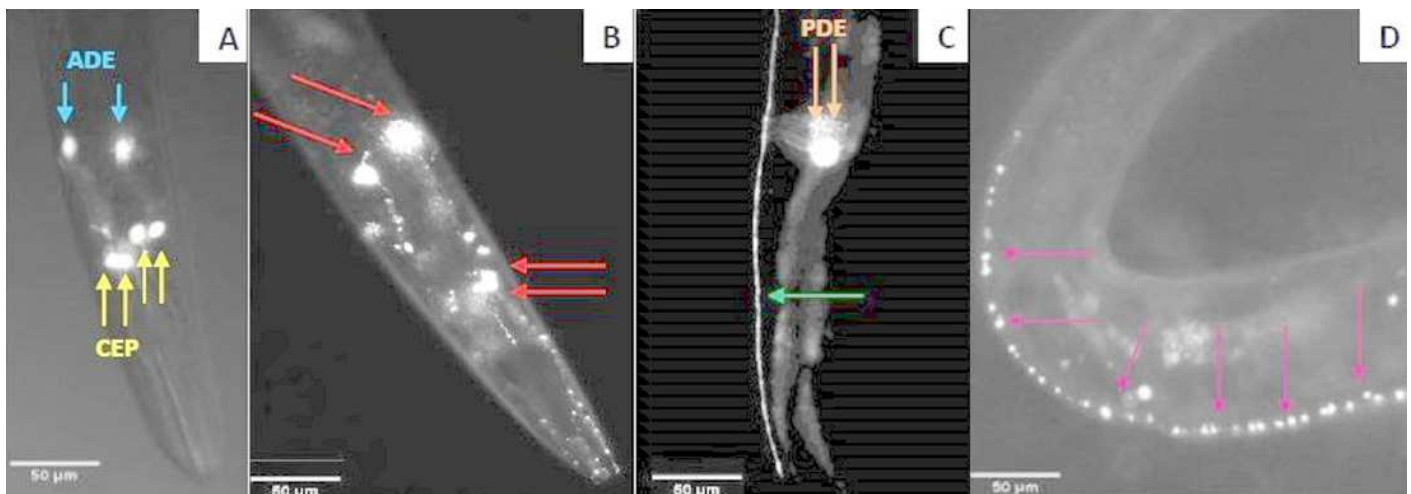


Figure 3

Effect of supernatants on *C. elegans* locomotion.

In (A) Worms of the wild-type strains N2 from synchronized eggs were raised in the presence or absence (control) of bacterial supernatants. In (B) Worms of the transgenic strain BY250 with GFP expression from synchronized eggs were raised in the presence or absence (control) of bacterial supernatants. The locomotion of each worm was examined by counting the number of body-bends per min ($n = 20/\text{treatment}$). Data are presented as the mean \pm SD. * $p < 0.05$.

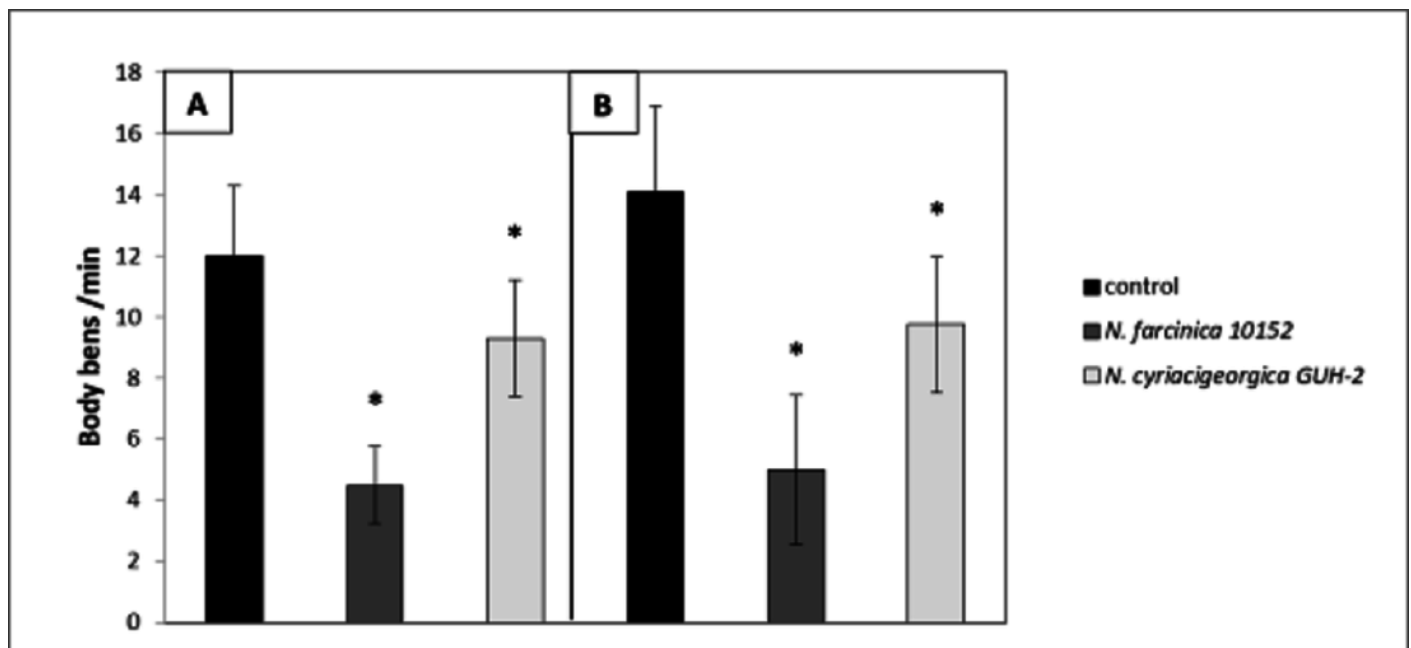


Figure 4

Histological observations on the mice who had died of sepsis after infection by *N. cyriacigeorgica* GUH-2.

Arrows indicate the presence of *Nocardia*. (A) Staining Fite on a kidney, *Nocardia* appears to multiply in a localized manner. (B) Hematoxylin and eosin staining of a kidney localizing *Nocardia* development.

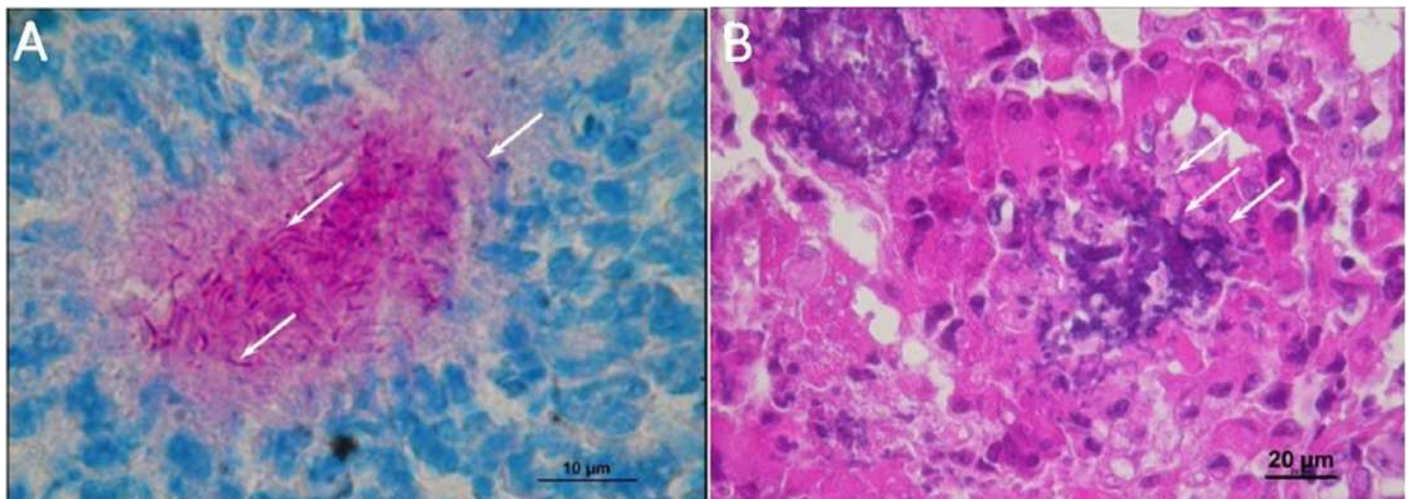


Figure 5

Histology of mice brains infected by *N. farcinica* 10152, with motor behavior disorders.

(A) Hematoxylin-eosin showing a focus of gliosis at the base of the forebrain in mice with rhythmic vertical movements of the head and hemiparesis. (B, C, D) Observations on mice brains with only hemiparesis. (B) Hematoxylin-eosin staining showing lymphocytic sleeves around capillaries (white arrow). (C) Fite staining showing the presence of *Nocardia* cells (black arrows) in the middle of apparently healthy neurons. (D) Immunohistochemical analysis revealed the presence of *Nocardia* antigens (brick red) surrounded by microglial cells.

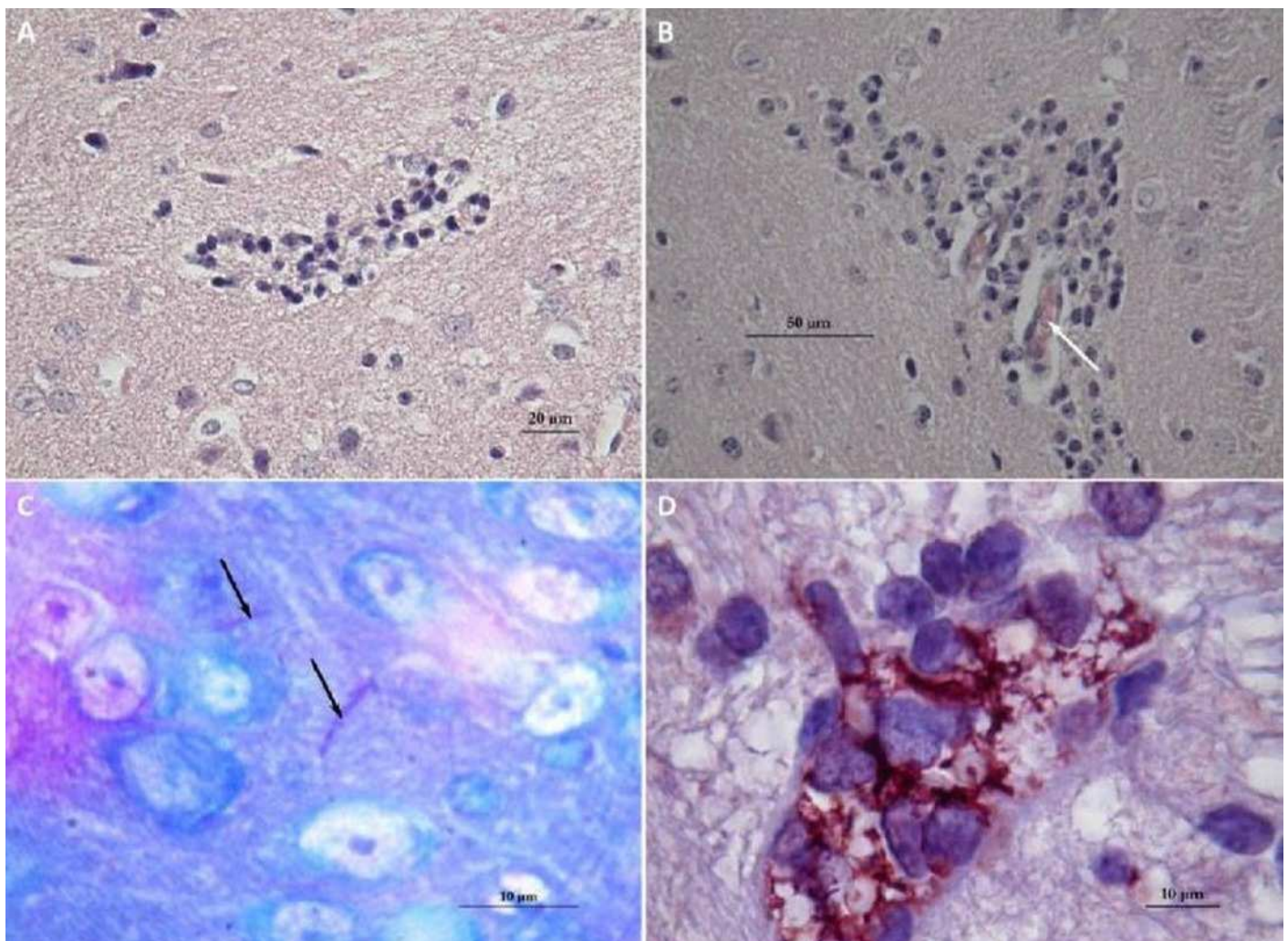


Table 1 (on next page)

Nocardia strains used in this study

Seven strains from different origins (clinical or environmental) were used in this study.

Strains tested on mice and nematodes are indicated.

Strains	Origin	Mouse experiment	Nematode experiment	Reference
<i>N. cyriacigeorgica</i> DSM 44484	Clinical	+	+	Yassin <i>et al.</i> , 2001
<i>N. cyriacigeorgica</i> OFN 04.100	Clinical		+	OFN's collection
<i>N. cyriacigeorgica</i> OFN 04.107	Clinical		+	OFN's collection
<i>N. cyriacigeorgica</i> GUH-2	Clinical	+	+	Beaman and Maslan, 1978
<i>N. cyriacigeorgica</i> OFN N27	Environmental		+	OFN collection
<i>N. farcinica</i> IFM 10152	Clinical	+	+	Ishikawa <i>et al.</i> , 2004
<i>N. asteroides</i> ATCC19247	Environmental	+	+	Gordon and Mihm, 1959

1

Table 2 (on next page)

Summary of nervous system damage observed in 242 worms infected with various *Nocardia* supernatants in Bennett medium at 10 days.

The percentages of affected *C. elegans* nematodes correspond to the number of nematodes having at least one dopaminergic neuron altered out of about 30 worms analyzed by fluorescence microscopy. Neuronal alteration was measured after 10 days of supernatant-nematode exposure. Nervous system damage was observed by fluorescence microscopy and can be summarized as: (i) blebbing, (ii) cell body rounding, and (iii) loss of neuronal bodies. Each strain was statistically compared with the negative control via the Fisher exact test (* $p < 0.05$).

Strains	Number of nematodes	Number of nematodes with damage to the nervous system			
		Blebbing	Cell body rounding	Neuronal body process loss	Total
Nematode culture control	30	1 (3.33%)	0 (0%)	1 (3.33%)	1 (3.33%)
Medium culture control	29	2 (6.9%)	1 (3.45%)	1 (3.45%)	2 (6.9%)
<i>N. cyriacigeorgica</i> DSM 44484	30	4 (13.33%)	2 (6.67%)	1 (3.33%)	4 (13.33%)
<i>N. cyriacigeorgica</i> 04.107	30	5 (16.67%)	0 (0%)	0 (0%)	5 (16.67%)
<i>N. asteroides</i> ATCC19247	31	8 (25.81%)	2 (6.45%)	0 (0%)	8 (25.81%)
<i>N. cyriacigeorgica</i> 04.100	30	7 (23.33%)	2 (6.67%)	0 (0%)	9 (30%)*
<i>N. cyriacigeorgica</i> N27	30	9 (30%)	5 (16.67%)	0 (0%)	10 (33.33%)*
<i>N. cyriacigeorgica</i> GUH-2	30	9 (30%)	8 (26.67%)	1 (3.33%)	11 (36.67%)*
<i>N. farcinica</i> IFM 10152	32	12 (37.5%)	12 (37.5%)	4 (12.5%)	17 (53.13%)*

1

Table 3(on next page)

Summary of behavioral disorders observed in 103 mice infected with different *Nocardia* strains.

Total affected mice correspond to the number of mice having at least one behavioral anomaly out of the 20 mice analyzed for each bacterial strain. Behavior anomalies were observed in mice after 13 days of infection and can be summarized by: (i) hemiparesis, (ii) vertical movement of the head, (iii) hemiparesis and trembling of the body, (iv) rigidity of movement, (v) death. The number of mice with abnormal behavior was indicated.

Strains	Dose	Number of mice	Number of deaths	Number of mice with neuronal anomalies ¹				Total
				Hemiparesis	Vertical movement of the head	Hemiparesis and body trembling	Rigidity of movement	
Medium culture control	-	6	0	0	0	0	0	0
<i>N. asteroides</i> 19247	Sub-lethal	17	0	0	0	0	0	0
<i>N. farcinica</i> 10152	Sub-lethal	20	0	4	1	4	0	9
<i>N. cyriaciogeorgica</i> 44484	Sub-lethal	20	0	2	3	0	2	7
<i>N. cyriaciogeorgica</i> GUH-2	Sub-lethal	20	0	2	0	0	0	3
<i>N. cyriaciogeorgica</i> GUH-2	lethal	20	13	1	2	0	0	3

1 ¹Total column corresponds to the affected number of mice having at least one behavioral disorder

2