1 Title: Pangenomic analysis reveals plant NAD+ manipulation as an important

2 virulence activity of bacterial pathogen effectors

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10 **Author Contributions:** W.M. and M.T.H. conceived the project. J.D.G.J contributed
11 to experimental design. M.T.H. did the experiments and analyzed the data. L.H
12 provided training and contributed to experimental design of LCMS. W.M., J.D.G.J and
13 M.T.H. wrote the manuscript.

17

Abstract

19 Nicotinamide adenine dinucleotide (NAD+) has emerged as a key component in

20 prokaryotic and eukaryotic immune systems and the recent discovery that

21 Toll/interleukin-1 receptor (TIR) proteins function as NAD+ hydrolases (NADase) link

22 NAD+-derived small molecules with immune signalling. We investigated pathogen

23 manipulation of host NAD+ metabolism as a virulence strategy. Using the pangenome

24 of the model bacterial pathogen *Pseudomonas syringae*, we conducted a structure-

25 based similarity search from 35,000 orthogroups for type III effectors (T3Es) with

26 potential NADase activity. Thirteen T3Es, including five newly identified candidates,

27 were identified that possess domain(s) characteristic of seven NAD+-hydrolyzing

28 enzyme families. Most *P. syringae* strains that depend on the Type III secretion system

29 to cause disease, encode at least one NAD+-manipulating T3E, and many have

30 several. We experimentally confirmed the type III-dependent secretion of a novel T3E,

31 named HopBY, which shows structural similarity to both TIR and adenosine

32 diphosphate ribose (ADPR) cyclase. Homologs of HopBY were predicted to be type

33 VI effectors in diverse bacterial species, indicating potential recruitment of this activity

34 by microbial proteins secreted during various interspecies interactions. HopBY

35 efficiently hydrolyzes NAD+ and specifically produces 2'cADPR, which can also be

36 produced by TIR immune receptors of plants and other bacteria. Intriguingly, this

37 effector promoted bacterial virulence, indicating that 2'cADPR may not be the
38 signalling molecule that directly initiates immunity. This study highlights a host-
39 pathogen battle ground centred around NAD^+ metabolism and provides insight into the
40 NAD^+-derived molecules involved in plant immunity.
41

Significance statement

43 NAD^+ metabolism plays a crucial role in plant and bacterial immunity. However, the
44 diversity and scope of NAD^+ processing steps in immune signalling remain unclear.
45 Furthermore, whether pathogens can manipulate NAD^+ metabolism to promote
46 virulence is unknown. By conducting a pangenomic screen of the plant pathogen *P.*
47 *syringae*, we found 13 type III effectors that potentially possess NADase activities,
48 indicating that NAD^+ manipulation is an important virulence mechanism. Further
49 characterization of a newly identified effector HopBY showed that it produces a cyclic
50 ADP-ribose isomer (2'cADPR) and promotes bacterial infection and symptom
51 development. This study clarifies the role of 2'cADPR in immune signalling and
52 provides an example of effectors as useful molecular probes to understand immunity.
53

Introduction

55 The metabolite and redox agent nicotinamide adenine dinucleotide (NAD^+) is a
56 universal co-factor and redox carrier involved in diverse cellular processes (1). Recent
57 research on the Toll/Interleukin-1 receptor (TIR) domain proteins has established a
58 central role of NAD^+ in immune signalling across biological kingdoms (2-4). Upon
59 activation, TIRs cleaves NAD^+ through a N-glycosidase activity to produce
60 nicotinamide (NAM), ADP-ribose (ADPR) and cyclic ADPR (5). It has recently been
61 shown that different TIRs produce distinct isomers of cADPR including 2'cADPR and
62 3'cADPR (6). These TIR NADase products are thought to activate immune signalling.
63 Plant and bacterial TIRs can make some of the same cADPR isomers and plant TIRs
64 can activate bacterial anti-viral immunity proteins *in vitro* (4). In addition to cADPR
65 isomers, other immunity-related products of plant TIR NADase and ADP-ribosylation
66 activities have recently been identified including pRib-AMP/ADP, ADPr-ATP and
67 diADPR (7, 8). Therefore, NAD^+-dependent immune signalling pathways are present
68 in plants and bacteria (4, 9).
69

2

70   In plants, the TIR domain is often associated with intracellular immune receptors that
71   also contain the nucleotide binding and leucine-rich repeat (NLR) domains (10). NLR
72   proteins detect pathogen effectors that have entered plant cells and can promote
73   disease. Once activated, TIR-NLR immune receptors trigger immunity through the
74   downstream Enhanced Disease Susceptibility 1 (EDS1)-helper NLR pathways (11).
75   Activated TIR domains have been shown to hydrolyse NAD^+ and produce various
76   products, including the cADPR isomer 2'cADPR (3, 6-8, 12). TIR-produced molecules
77   pRib-AMP/ADP, ADPr-ATP and diADPR have been shown to bind to and activate
78   specific EDS1-helper NLR complexes (7, 8), potentially linking NADase activity of
79   activated TIR proteins to EDS1-dependent immune signalling. In addition, a TIR-only
80   resistance protein in plants, RBA1, exhibits 2',3'-cNMP synthase activity *in vitro* and
81   these cyclic nucleotides may also contribute to immunity (13).
82
83   Since NAD^+ metabolism contributes to innate immunity, we hypothesized that
84   manipulation of this process by pathogens might promote virulence. Pathogens rely
85   on effector proteins to manipulate host cellular processes, and effectors with NADase
86   activities have been reported. The bacterial animal pathogen *Brucella abortus*
87   produces TIR effectors BtpA and BtpB, which promote infection by reducing NAD^+
88   levels in the animal host and BtpA has also been shown to produce 2'cADPR (5, 14).
89   The plant pathogen *Pseudomonas syringae* produces a type III effector (T3E)
90   HopAM1 that also has a TIR domain. HopAM1 cleaves NAD^+ and produces 3'cADPR,
91   which was proposed to be responsible for the immune suppression activities of
92   HopAM1 during infection (6, 15). In addition, the bacteriophage protein Tad1
93   sequesters the TIR-produced molecule 2'cADPR and likely also 3'cADPR to overcome
94   anti-viral defence of the bacterial host (6, 9, 16). In addition to producing small
95   molecules from NAD^+ cleavage that might interfere with host signalling pathways, the
96   NAD^+ reduction activities of these effectors may directly contribute to host cell death
97   (14).
98
99   Enzymatic cleavage of NAD^+ can occur at either the pyrophosphate bond or the N-
100   glycosidic bond (Fig 1A). Pyrophosphate bonds can be cleaved by NUDIX
101   (nucleoside diphosphate linked to a variable moiety X) pyrophosphatases (17) and
102   phosphodiesterases (PDEs). The effector Avr3b produced by the oomycete pathogen
103   *Phytophthora sojae* harbors a NUDIX domain and can hydrolyse NADH, ADPR and

3

104   2',3'-cNMPs (13, 18). Enzymes that can cleave the N-glycosidic bond, apart from the

105   TIR proteins, include ADP-ribosyltransferases (ART), nucleoside hydrolases (NH),

106   ADPR cyclases, and sirtuins (Fig 1A). ARTs cleave NAD^+ to release ADPR, which is

107   often used for post-translational modifications of proteins and nucleic acids (19, 20).

108   ART effectors have been identified in various pathogens although most of them are

109   presumed to modify host protein targets (21). However, some ARTs act primarily as

110   NAD^+ glycohydrolases and don't require a protein substrate (22). Conceivably, at least

111   some ART effectors may function to manipulate NAD^+ metabolism. The *P. syringae*

112   T3E HopQ1 and the related *Xanthomonas euvesicatoria* T3E XopQ1 are nucleoside

113   hydrolases. HopQ1 was shown to alter purine metabolism (23) and activate cytokinin

114   signalling (23, 24). XopQ1 was also recently shown to hydrolyse 2',3'-cAMP (13). So

115   far, pathogen effectors with sirtuin or ADPR cyclase domains have not been identified.

116

117   Here, we employ pangenomic and structure-based similarity searches to identify

118   effectors with potential NADase activity from the species complex of the model

119   bacterial pathogen *P. syringae*. *P. syringae* relies on T3S to delivery effectors into

120   plant cells that suppress immunity and promote disease symptoms. We found *P.*

121   *syringae* encodes T3Es with six of the seven enzymatic activities that can hydrolyze

122   NAD^+, suggesting that manipulation of NAD^+ metabolism is an important virulence

123   mechanism. We then focused on a novel T3E, named HopBY, that possesses an

124   ADPR cyclase activity and characterized its potential virulence function. Intriguingly,

125   HopBY hydrolyzes NAD^+ and produces 2'cADPR *in vitro* and during infection.

126   2'cADPR can also be produced by plant TIR-NLRs, but our results indicate that it is

127   unlikely to be an activator of immune signalling.

128

129   **Results**

130   **Identification of NADases from the *P. syringae* pangenome**

131   The *P. syringae* species complex has been divided into phylogroups, which broadly

132   correspond to phylogenetic "species" sharing 95% Average Nucleotide Identity (ANI)

133   (25). Plant pathogens mainly belong to the monophyletic clade of "primary"

134   phylogroups and rely on a canonical Hrp1 type III secretion system (T3S) to cause

135   disease (26). The "secondary" phylogroups include environment-associated strains

136   which may not possess the Hrp1 T3S and encode very few T3Es when they do (27,

137   28).To examine NADase-encoding proteins across the species complex, 531 *P.*

4

138 *syringae* genomes were analysed. A phylogeny based on the core genome grouped
139 the strains into ≥ 95% identical ANI groups which loosely correspond to previously
140 defined phylogroups (Fig S1). Orthology analysis assigned the pangenome of
141 2,867,287 protein-encoding genes into 23,583 orthogroups (OGs), with an additional
142 11,608 singletons. In total, 35,191 OGs were further screened for candidate NADase
143 domains.

144

145 Two complementary approaches (HMMER and HHsearch) were employed to predict
146 proteins that may harbour any of the seven enzymatic domains related to NAD$^+$
147 hydrolysis (Pipeline illustrated in Fig S2A). These domains include ART, NUDIX, PDE,
148 TIR, sirtuin, ADPR cyclase, and nucleoside hydrolase (Fig 1A; Table S1). The
149 HMMER-based approach employed a Hidden Markov Model to identify any proteins
150 with sequence similarity to pfam seed alignments for each enzyme group. Using this
151 prediction method, 146 OGs were identified as candidate NADases. The HHsearch-
152 based approach used orthogroup sequence alignments with secondary structure
153 information. Based on HMM-HMM comparison with the PDB database, HHsearch
154 picked up 564 OGs as candidate NADases. Importantly, 136 of the 146 candidates
155 identified by HMMER were also identified by HHsearch (Table S2, full details in Table
156 S3), testifying the robustness of the structure-based prediction. When mapping on the
157 core genome phylogeny, the total of 574 candidate NADases are distributed across
158 the *P. syringae* species complex in both primary and secondary phylogroups without
159 a clear pattern in relation to pathogenesis (Fig S2B). This is consistent with their role
160 in regulating housekeeping processes and anti-viral immunity.

161

162 **Prediction of NADases as Type III effectors**
163 From the 574 candidate NADase OGs, we further identified T3Es because they are
164 major virulence factors in *P. syringae*. Our prediction pipeline included an initial screen
165 using the program EffectiveT3 (29) followed by a promoter search for the *hrp* box,
166 which is bound by the T3S-specific alternative sigma factor HrpL (30). The candidates
167 identified through this pipeline were further examined on their N-terminal amino acid
168 sequences for characteristics related to T3S-dependent secretion (31). The outcome
169 of this analysis revealed 13 NADase OGs as high confidence T3Es (Table 1; full list in
170 Table S4; Fig S3-S4). The robustness of this analysis is verified by the successful

171 identification of all the known T3Es with NADase-encoding domains including the ART
172 effectors HopF/HopBB, HopO1, AvrRpm, HopS, and HopU1, the NUDIX effector
173 HopAG1, the NH effector HopQ1, and the TIR effector HopAM1. The detection of
174 multiple non-overlapping homologies to different enzymes in the same protein also
175 revealed that HopAG1 carries a previously unknown ART domain on the N-terminus
176 in addition to the NUDIX domain on the C-terminus. Importantly, we identified five new
177 NADase T3E candidates, including two ARTs (OG5256, OG18195), one PDE
178 (OG4578), one TIR (OG8567), and one ADPR cyclase (OG18056).

179

180 The distribution of the 13 known NADase T3Es and new candidates was mapped on
181 the core genome phylogeny of *P. syringae* species complex (Fig 1B). A clear
182 difference between the primary and secondary phylogroups can be visualized, with
183 known and predicted NADase T3Es almost exclusively encoded by strains belonging
184 to the primary phylogroups, consistent with their potential role in host manipulation
185 (Fig 1B and Fig S5). 93% of the primary phylogroup strains have at least one NADase
186 T3Es and 72% possess T3Es belonging to more than one enzyme family. On average,
187 each primary phylogroup strain encodes three T3Es that potentially hydrolyze NAD^+.
188 NAD^+ hydrolysis is therefore a common virulence mechanism utilized by *P. syringae*
189 pathogens.

190

191 **OG18056 is a NADase with structural homology to both ADPR cyclases and**
192 **TIRs**
193 Considering the essential role of NAD-derived cADPR isomers in plant immunity, we
194 were particularly interested in a novel T3E candidate OG18056, which is predicted to
195 have an ADPR cyclase domain that has not been found in pathogen effectors. ADPR
196 cyclase shares a similar enzymatic activity with TIR and can also hydrolyze NAD^+ to
197 produce cADPR (32). Using AlphaFold2 (33), we generated a structural model for
198 OG18056. Superimposition of this model with the human ADPR cyclase CD38 showed
199 an RMSD value of 2.94 Å. The structural model of OG18056 also showed similarity
200 with the TIR domain of the human SARM1 protein with an RMSD value of 2.96 Å (Fig
201 2A). These results suggest that OG18056 possesses a fold that resembles both TIR
202 and ADPR cyclase, although our initial prediction only revealed similarity with ADPR
203 cyclase through HHsearch structure-based analysis.

204

6

205 The NAD$^+$ hydrolysis activity requires a catalytic glutamate (Glu, E) in TIR and ADPR

206 cyclase. Structural alignment revealed E305 in OG18056 to be located at the

207 corresponding position of the catalytic E642 in hSARM1 (2) as well as E226 in hCD38

208 (34) (Fig 2B). Furthermore, a ligand-binding pocket, containing the putative catalytic

209 residue E305, was predicted in OG18056 3D model using the program P2RANK with

210 a probability of 0.9 (35). Molecular docking revealed that NAD$^+$ could fit into this

211 predicted pocket with a binding affinity of -8.76 kcal/mol (Fig 2C). These results are

212 consistent with the hypothesis that OG18056 is an active NAD$^+$-cleaving enzyme.

213

214 To evaluate the enzymatic activity of OG18056, we used HPLC-MS analysis to detect

215 NAD$^+$ hydrolysis and production of various form(s) of cADPR. In a semi-*in vitro* assay

216 (36), 3xFLAG-tagged OG18056 and its predicted catalytic mutant OG18056^{E305A} were

217 expressed in *Nicotiana benthamiana* by *Agrobacterium*-mediated transient

218 expression. Wild type and mutant OG18056 proteins were immunoprecipitated and

219 incubated with NAD$^+$. The resulting metabolites were extracted and analyzed by

220 LCMS. The results show that OG18056 produced a peak for m/z of 542.06, which is

221 consistent with that of cADPR, at a retention time of ~3.0 minutes (Fig 2D).

222 OG18056^{E305A} did not produce this compound. The proteins were confirmed to be

223 expressed and immunoprecipitated (Fig 2E). These results demonstrate that

224 OG18056 is indeed an active NADase that produces a mass corresponding to cADPR.

225

OG18056 (HopBY) produces 2'cADPR during bacterial infection

227 To assess whether OG18056 is a secreted protein dependent on T3S, we tested its

228 expression pattern and T3S-dependent secretion using a minimal medium (Fig 3A).

229 The gene encoding OG18056, tagged at the C-terminus with a HA epitope, was

230 introduced into *P. syringae* pv. tomato strain DC3000 (PstDC3000) under its native

231 promoter. Bacterial cells were induced in a *hrp*-minimal medium (HM) and OG18056

232 proteins were detected in the cell culture supernatant by western blotting. We

233 observed that OG18056 had a minimal expression in the rich medium KB, but was

234 drastically induced in the HM medium, an expression pattern commonly observed in

235 T3S-related genes. Consistent with the detection of a *hrp* box in the promoter of

236 OG18056 gene, this result shows that OG18056 is regulated by T3S. Furthermore,

237 OG18056 proteins were detected in the supernatant of PstDC3000 cell culture, but not

238 in the supernatant of the Δ*hrcC* mutant of PstDC3000, which does not form a functional

239   T3S injectosome. These results suggest that OG18056 is likely a T3E that can be

240   secreted in a T3S-dependent manner. We therefore name this new T3E HopBY based

241   on the *P. syringae* T3E nomenclature system (28, 37).

242

243   We next examined whether HopBY hydrolyzes NAD^+ and produces isomer(s) of

244   cADPR during natural infection. HopBY or HopBY^{E305A} was expressed in the D36E

245   mutant of PstDC3000 under its native promoter (Fig S6). D36E has the intact T3S but

246   lacks all 36 known T3Es, and is not pathogenic (38). The transformed bacteria were

247   then used to inoculate *Arabidopsis thaliana.* Eighteen hours post-infiltration,

248   metabolites were extracted from the inoculated leaf tissues and subjected to LCMS

249   analysis. We detected a clear peak corresponding to m/z 542.06 at the same retention

250   time (~3 min) as in the semi-*in vitro* assay (Fig 3B). This peak was absent in leaves

251   inoculated with D36E carrying the empty vector or expressing the catalytic mutant

252   HopBY^{E305A}, indicating that it is the product of HopBY NADase activity. As a control,

253   leaves inoculated with D36E expressing HopAM1 showed a peak with a retention time

254   of ~4.5 minutes, corresponding to 3'cADPR, as previously reported (6, 15). To

255   determine which isomer of cADPR was produced by HopBY, we ran LCMS on a cell

256   lysate of *E. coli* expressing AbTIR, which is produced by the opportunistic bacterial

257   pathogen *Acinetobacter baumannii*. AbTIR is known to produce 2'cADPR (6).

258   Interestingly, the HopBY product had the same retention time as the AbTIR product

259   (Fig 3C). When running a mixture of the different cADPR samples, a single peak with

260   m/z of 542.06 and a retention time of ~3.0 minutes appeared, suggesting that the

261   NAD^+ hydrolysis product of HopBY is likely the same or very similar to AbTIR, i.e.

262   2'cADPR (Fig 3D). Comparison of MS2 fragmentation profiles demonstrated that the

263   peak produced by HopBY generated similar fragment ions to the cADPR standard and

264   cADPR variants produced by other NADases, confirming that the HopBY product was

265   indeed a cADPR (Fig S7).

266

267   To determine if HopBY has any impact on overall NAD^+ levels during infection, we

268   measured the abundance of NAD^+ at days 0 and day 3 after inoculation with D36E

269   carrying either HopBY or HopAM1 (5 x 10^6 CFU/mL). NAD^+ levels showed a small but

270   significant reduction in leaf tissues inoculated with HopBY-expressing bacterium at

271   day 3 compared to day 0 (Fig. S8). This reduction was not observed in tissues

8

272   inoculated with D36E expressing HopAM1 or carrying the empty vector. These results

273   suggest that HopBY may reduce NAD$^+$ levels in host cells as a virulence mechanism.

274

275   **HopBY promotes bacterial infection and necrosis**

276   We next examined the potential virulence activity of HopBY during bacterial infection.

277   D36E expressing HopBY or the catalytic mutant HopBY^{E305A} was used to inoculate *A.*

278   *thaliana* plants at a dose of 5 x 10^6 CFU/mL. We observed chlorotic symptoms in

279   leaves inoculated with D36E expressing HopBY, but not HopBY^{E305A}, after three days

280   (Fig 4A). When inoculated at a higher dose (1 x 10^8 CFU/mL), tissue collapse

281   appeared at 24 hours post inoculation, which is similar in appearance to immunity-

282   related hypersensitive response triggered by effectors recognized by NLRs, such as

283   AvrRps4 (Fig 4B). It is noteworthy that D36E expressing AvrRps4 did not induce the

284   chlorosis at the lower inoculum as HopBY did (Fig 4A). Similar to chlorosis, the cell

285   death-like phenotype was dependent on the NADase activity as the HopBY^{E305A}

286   mutant failed to induce this symptom. In addition, inoculation of *Pst* DC3000Δ*hrcC*

287   expressing HopBY did not cause these symptoms, which is consistent with the

288   previous results suggesting that HopBY is likely translocated during bacterial infection

289   in a T3S-dependent manner (Fig. S9).

290

291   Because 2'cADPR can be produced by some plant TIR-NLRs, it is therefore a

292   possibility that HopBY may activate an immune response, which would lead to cell

293   death due to hypersensitive response. To examine this, we inoculated D36E

294   expressing HopBY1 in the *A. thaliana* mutant *eds1-2*, which is critical for TIR-NLR-

295   mediated immune signalling (39). The results show that HopBY was still able to induce

296   the chlorosis (Fig 4A) and cell death-like phenotypes in the *eds1* mutant (Fig 4B). In

297   contrast, the cell death induced by AvrRps4 as a hypersensitive response was

298   abolished in *eds1-2* (Fig 4B). To further confirm HopBY did not trigger immunity in *A.*

299   *thaliana*, we evaluated the *in planta* bacterial population at three days post inoculation.

300   While expression of AvrRps4 caused a decline in bacterial population, HopBY actually

301   promoted infection (Fig 4C). This virulence activity was not observed in the catalytic

302   mutant HopBY^{E305A}. These results demonstrate that HopBY enhances bacterial

303   infection by promoting bacterial colonization and symptom development. These

304 virulence functions rely on its NADase activity, and possibly related to the production
305 of 2'cADPR.

306

307 **HopBY homologues are associated with T3 and T6 secretion systems**
308 HopBY is only present in three *P. syringae pv. coronafaciens* strains, which are
309 pathogens of grass species. The *hopBY* gene is flanked by transposase genes and
310 nearby genes encoding conjugation machinery (*virB* homologues). It is within 15 Kb
311 of T3E homologues *hopV1 and hopAA1* as well as a coronatine biosynthesis operon,
312 which also contributes to virulence (Fig S10A). This genomic location suggests that
313 *hopBY* may have been acquired via horizontal gene transfer. A skew in GC content
314 further indicates that this genomic region may represent a pathogenicity island (Fig
315 S10A).

316

317 Using BLASTP, we identified homologs of HopBY in diverse bacterial lineages ranging
318 in amino acid sequence identity from 26%-100%. HHsearch confirmed that these
319 homologs all have an ADPR cyclase-like fold on their C terminus (Table S5).
320 Examination of the genomic region surrounding all homologs revealed they are often
321 associated with transposons, indicating a strong signature of horizontal gene transfer
322 (Fig S10B). A phylogeny based on structure-guided protein sequence alignment
323 grouped these HopBY homologs into two clades (Fig 5A). The HopBY from *P. syringae*
324 is grouped with homologs from other *Pseudomonas* spp. as well as plant pathogenic
325 Enterobacteriaceae species within the genera *Brenneria* and *Pectobacterium*. All
326 these genomes contain genes encoding a Hrp1 T3S, the canonical T3S found in *P.*
327 *syringae*. Importantly, the homologous genes in this clade are all preceded by a *hrp*-
328 box promoter (Table S5), indicating that they are likely T3Es. In the *Brenneria* (n=2)
329 genomes, the *hopBY* homologous genes are present close to the T3S machinery gene
330 clusters, further supporting their identity as T3Es. The other clade includes more
331 distinct HopBY homologs including one in *Paraburkholderia* sp. ZP32-5, which was
332 also predicted as a possible T3E. *Paraburkholderia* sp. ZP32-5 contains the Hrp2 type
333 of T3S machinery and the HopBY homolog gene is located in close proximity to the
334 T3S gene cluster. Upstream of this gene there are PIP-box motifs, which are
335 associated with Hrp2 T3S-regulated gene expression. Therefore, this HopBY homolog
336 is likely also a T3E. Interestingly, other homologs in this clade are often located
337 adjacent to type VI secretion system (T6S) genes such as *vgrG*, *tssA*, *vasA* and *tssG*.

10

338 T6S is an important secretion system for bacterial interactions with other bacteria and

339 eukaryotes by delivering toxins that kill recipient cells (40). Indeed, these more distant

340 homologs of the *P. syringae* HopBY were predicted to be putative T6S effectors. Some

341 of these proteins have domains such as the N-terminal Rhs (Rearrangement Hotspot)

342 core domain, which are often found in T6Es (41).

343

344 Using structure models predicted by AlphaFold2 (Fig 5B), we aligned the predicted

345 ADPR cyclase domain in all the homologs to the *P. syringae* HopBY, which confirmed

346 their structural similarity with RMSD values ranging from 0 - 4.44 Å (Table S5). We

347 then aligned the region surrounding the catalytic residue E305 of *P. syringae* HopBY.

348 This revealed that the glutamate as well as an upstream tryptophan, which was shown

349 to be important for cyclization of the NAD^+ hydrolysis products (6, 32), are highly

350 conserved in all homologs, indicating they may be active enzymes (Fig 5C). The

351 existence of HopBY ADPR cyclase domain homologs across effectors from T3 and T6

352 secretion systems indicates this enzymatic activity may have been adopted by distinct

353 secretion systems that function in interspecies interactions.

354

Discussion

356 Exploiting the pangenomic diversity and the extensive genome sequence resources

357 of *P. syringae*, we conducted a comprehensive search to identify T3Es with enzymatic

358 activities that can hydrolyse NAD^+. This revealed host NAD^+ manipulation as a

359 virulence strategy in a model plant pathogen. *P. syringae* encodes a diverse set of

360 T3Es with six different enzymatic activities, which potentially modulate NAD^+

361 metabolism in plant cells during infection. Thus, manipulation of NAD^+ and NAD^+-

362 related molecules is an important virulence mechanism commonly used by diverse *P.*

363 *syringae* strains and likely by other plant and animal pathogens as well as

364 bacteriophages.

365

366 NAD^+ is a key metabolite that is involved in a wide range of cellular processes. NAD^+

367 depletion could lead to cell death (4). Therefore, it is possible that effectors contribute

368 to tissue damage through NAD^+ depletion, which may be related to the development

369 of disease symptoms. Indeed, the new T3E HopBY identified in this study caused

370 chlorosis and tissue collapse. HopBY delivery by D36E led to a moderate, but

371 statistically significant reduction in NAD^+ abundance in infected tissue (Fig S8). Since

11

372   not all plant cells in the leaf will receive effector delivery during infection, the observed
373   reduction in overall NAD$^+$ level may indicate significant depletion in those cells that do
374   receive HopBY. Conceivably, HopBY-associated necrotic symptoms is due to NAD$^+$
375   depletion in these cells. HopBY-associated phenotypes are EDS1-independent,
376   indicating that they are not due to activation of EDS1-mediated plant immunity but may
377   be a result of NAD$^+$ depletion. Overexpression of the TIR domain of AbTIR and some
378   plant TIRs in *N. benthamiana* also cause cell death independent of EDS1 (9, 42). This
379   suggests alternative immune signalling pathways may exist, or that these TIR proteins
380   may cause NAD$^+$ depletion when accumulated to a high level.

381

382   In addition to the potential impact of NAD$^+$ consumption activities, NADase effectors
383   could also promote disease through the production of specific metabolites. The newly
384   emerged role of NAD-derived small molecules in immune signalling leads to a
385   possibility in which pathogens can use NADase effectors to suppress immunity. We
386   can speculate that pathogen effectors could compete with host immune proteins for
387   substrates (43). In addition to NAD$^+$, the enzymatic domains identified from the *P.*
388   *syringae* T3Es may also use other molecules such as ADPR and cADPR as
389   substrates. Therefore, hydrolysis and depletion of immune signal molecules or their
390   precursors could be a virulence mechanism. Although these hypotheses remain to be
391   tested, investigation of the enzymatic activity of NADase effectors will offer a unique
392   opportunity to dissect immune signalling through NAD-derived small molecules.

393

394   TIR proteins across biological kingdoms generate cyclic nucleotide products that play
395   vital roles in immune signalling (3, 4). *P. syringae* produces HopAM1 as a TIR effector
396   and HopBY shows structural and functional similarity to both TIR and ADPR cyclase.
397   Both HopAM1 and HopBY could promote bacterial colonization indicating that they
398   could either suppress plant immunity (15) and/or promote an environment suitable for
399   disease progression. It is intriguing that plant and pathogen NADase activities can
400   result in opposite phenotypes (resistance vs susceptibility). An attractive possibility is
401   that these TIR/ADPR cyclase effectors produce distinct isomers that can activate
402   immune signalling. Indeed, HopAM1 produces 3'cADPR, distinct from the 2'cADPR
403   detected from the NAD$^+$ hydrolysis reaction of activated plant TIR proteins (3) and
404   could function as a negative regulator of plant immunity (6, 15). However, the
405   somewhat surprising finding that HopBY produced a significant amount of 2'cADPR

406 during bacterial infection reflects the complexity of NAD$^+$ metabolism in immune
407 response.

408

409 A role for 2'cADPR in TIR-NLR initiated immune signalling has been hypothesized but
410 not formally demonstrated (3). Several previous observations suggest that 2'cADPR
411 production alone is insufficient to activate immune signalling. A fusion receptor with
412 the bacterial AbTIR and a mammalian NLR did not activate cell death when expressed
413 in *N. benthamiana*, whilst several plant TIR-mammalian NLR fusions did (36).
414 Furthermore, expression of bacterial TIRs alone led to 2'cADPR production but did not
415 activate EDS1-mediated immunity in plants (4, 9). Finally, recent *in vitro* studies show
416 that EDS1-helper NLR complexes directly bind to pRib-AMP, pRib-ADP, ADPr-ATP
417 and diADPR. Therefore, although 2'cADPR can be produced by plant TIRs, it appears
418 insufficient to directly initiate EDS1-mediated immune signalling.

419

420 Homologous proteins to HopBY were identified across diverse bacterial lineages. The
421 *P. syringae* allele is closely related to other predicted T3Es from plant pathogens, but
422 the other homologs were predicted as T6Es, which are involved in bacteria-bacteria
423 and bacteria-eukaryotic interactions (40). NADase T6Es have been discovered before.
424 Two ART-like families of T6Es, Tse6 and Tne2, were involved in bacteria-bacteria
425 competition. Injecting into the recipient cells, Tse6 and Tne2 deplete NAD$^+$, leading to
426 cell death (45, 46). This mechanism is reminiscent to bacterial anti-viral immunity
427 which involves TIR signalling to activate the sirtuin ThsA to deplete NAD$^+$. It is
428 intriguing that HopBY homologs are widespread in bacteria as T6Es. Testing whether
429 these T6Es can function as cytotoxins in bacteria-bacteria or bacteria-eukaryote
430 competitions will be an interesting future experiment. Presumably, the bacterial strains
431 possessing T6 effectors related to HopBY must also encode an immunity protein or
432 mechanism to prevent the action of the T6 effector before secretion (46). Some of the
433 HopBY homologous T6Es contain the N-terminal Rhs domain (47). Rhs allows
434 diversification of T6Es at the C-terminal toxin domains via recombination (41). The
435 HopBY NADase domain likely recombined into these Rhs loci, generating a new
436 virulence function. Similarly, the HopBY homologous T3Es could also be created
437 through fusion to the T3S-dependent promoter and N-terminal secretion sequence via
438 the terminal reassortment mechanism (48). Therefore, the HopBY domain has been
439 incorporated into effectors for different interspecies competitions using distinct

13

440   secretion system machinery, demonstrating the importance of NAD$^+$ manipulation in

441   these antagonistic interactions.

442

443   Much remains to be discovered about the diverse NADase activities of TIR domains

444   and the complexity of their cyclic nucleotide products. The prevalence of pathogen

445   effectors with NADase activities testifies to the importance of NAD$^+$ metabolism as a

446   battleground in host-pathogen interactions. Understanding how effectors with NAD$^+$

447   hydrolysis activities promote virulence, and if and how they can disrupt immune

448   signalling pathways in plant hosts, will continue to provide new insights into immune

449   signalling and its circumvention in bacteria, animals, and plants.

450

451   **Methods**

452   Standard procedures used in this manuscript are documented in detail in the

453   Supplementary Methods. Specific methods developed here are included below.

454

455   **NADase T3E prediction**

456   The pipeline to identify nucleosidase candidates is presented in Fig S2A. For HMMER

457   (http://hmmer.org/) analysis, Pfam seed alignments for enzyme families (listed in Table

458   S1) were obtained from Pfam (http://pfam.xfam.org/) (downloaded on December 1st,

459   2020). The script hmmbuild was used to create a HMM profile for each seed alignment.

460   The command hmmsearch was used to screen all *P. syringae* proteins. HMMER

461   results were filtered based on a tolerant bit-score of $\geq$ 15 (49) and grouped by

462   orthogroup. Orthogroups with positive hits were further filtered to remove those in

463   which less than 5% of the orthogroup members were a hit as these rare hits may not

464   represent the orthogroup function.

465

466   HHsearch (50) was also used for each orthogroup to predict similarity to proteins in

467   the PDB database (downloaded on October 28th, 2021). First, a multi-sequence

468   alignment (MSA) was generated using ClustalW and a consensus sequence for each

469   orthogroup was generated using hhcon. Next, the secondary structure predicted by

470   PSIPRED was added to the alignment (51) before running HHsearch. Bash scripting

471   was used to extract candidates with hits towards NADase enzymes in the database

14

472   using a cutoff of $\geq$ 80% probability score. Two additional hits per protein with non-

473   overlapping regions were also identified.

474

475   Candidate orthogroups predicted with HMMER or HHsearch were next screened with

476   EffectiveT3 (29). Orthogroups containing hits with a score $\geq$ 0.9 were further analyzed

477   for putative *hrp* box sequences in their promoter region (within 1000 bp upstream of

478   the protein-coding sequence) using FIMO (30) based on the *hrp* box sequence

479   GGAACC(15-17N)CCACNNA as input.

480

481   Candidate T3Es were manually filtered based on the percentage of sequences within

482   the orthogroup predicted to be an effector ($\geq$ 50%) and the percentage of these

483   sequences that contained a possible *hrp*-box promoter ($\geq$ 25%). Orthogroups only

484   present in genomes without a predicted Hrp1 T3S were removed. Candidates T3Es

485   were then manually examined to determine if they had characteristic N-terminal

486   characteristics of *P. syringae* T3Es (31, 52). This included 1) a high percentage of

487   serines in the first 50 amino acids; 2) an aliphatic amino acid in positions 3 or 4; and

488   3) a lack of negatively charged residues within the first 12 positions. Genomic locations

489   of each T3E candidate gene were also manually examined using Geneious Prime

490   2022.0.1 to examine gene neighbourhoods (https://www.geneious.com).

491

492   **HPLC-MS**

493   Cyclic-ADP ribose was analysed using a 1290 Infinity UHPLC system equipped with

494   a 6546 Q-ToF (Agilent). Separation was on a 100×2.1mm 2.6µ Kinetex EVO C18

495   column connected in series to a 100×2.1mm 2.6µ Kintex F5 column (both

496   Phenomenex) using the following gradient of methanol (solvent B) versus 0.1% formic

497   acid adjusted to pH 6.0 with ammonium hydroxide (solvent A), run at 0.27 mL.min-1

498   and 35°C: 0 min, 0% B; 1.5 min, 3% B; 4.5 min, 15% B; 6.0 min, 60% B; 6.5 min, 95%

499   B; 7.5 min, 95% B; 7.6 min, 0% B; 11.6 min, 0% B. Detection was by positive

500   electrospray ionisation using the dual jet-stream ESI source. The instrument collected

501   full MS from m/z 100-1700 at 6 spectra per second, and automatic MS/MS of the two

502   most abundant precursors at 8 spectra per second, with an isolation width of m/z 4.0

503   ("medium") and 35% collision energy. Each precursor was ignored for 0.2 min, in

504   favour of less abundant precursors, after two MS/MS spectra had been collected.

505   Spray chamber conditions were 8 L.min-1 drying gas at 320°C, 35 psi nebulizer
506   pressure, 11 L.min-1 sheath gas at 350°C, 3500 Vcap, 1000V nozzle voltage, 175V
507   fragmentor, 65V skimmer, and 750V Octopole RF. The instrument was calibrated
508   according to the manufacturer's instructions before use, and reference masses
509   (121.0509; 922.0098) were used during the run to ensure mass accuracy.
510

518

519   **Data and material availability:** All code are available at https://github.com/michhulin/
520   Pseudomonas. Materials are available from W.M. upon request under a materials
521   transfer agreement with the Sainsbury Laboratory.
522

523 **Table 1. Known NADase T3Es and predicted candidates in *P. syringae*.**

Group	Ortho-group	No. genes	No. effectors	% effectors	No. *hrp* promoter	% *hrp* promoter	No. genomes	No. T3S+ genomes	% T3S+ genomes	Notes	Secretion signal score (out of 3)
ART	4303	339	332	98	299	90	188	187	99	HopF/BB	2
ART	5256	144	130	90	38	29	37	35	95	**Novel**	**3**
ART	5397	133	91	68	54	59	46	46	100	HopO1	3
ART	5686	112	103	92	91	88	87	87	100	AvrRpm	3
ART	6340	69	54	78	53	98	53	53	100	HopS	3
ART	9885	14	14	100	10	71	10	10	100	HopU1	3
ART	18195	3	3	100	3	100	3	3	100	**Novel**	**3**
ART[a]	4463	297	223	75	213	96	213	213	100	HopAG1	3
NH	4871	195	124	64	121	98	118	117	99	HopQ1	2
NUDIX	4463	297	223	75	213	96	213	213	100	HopAG1	3
PDE	4578	258	214	83	102	48	101	100	99	**Novel**	**2**
TIR	6638	55	49	89	45	92	38	37	97	HopAM1	3
TIR	8567	23	21	91	15	71	14	14	100	**Novel**	**2**
ADPR cyclase	18056	3	3	100	3	100	3	3	100	**Novel**	**3**

524
525 No. genes: number of genes in orthogroup.

526 No. effectors: number of genes within orthogroup that were predicted to encode an effector.

527 % effectors: % orthogroup that may encode effector.

528 No. *hrp* promoter: Number of predicted effector genes per orthogroup that had a predicted *hrp* box

529 % *hrp* promoter: % effector genes in orthogroup that had *hrp* promoter.

530 No. T3S+ genomes: Number of genomes encoding the genes in each orthogroup predicted as

531 effectors with a *hrp* box which have the genes required for Hrp1 T3S machinery.

532 % genomes with Hrp1 T3S: % of effector genes with a *hrp* box per orthogroup that were in genomes

533 encoding Hrp1 T3S.

534 Secretion signal score: A score from 0-3 based on three previously defined criteria of T3E secretion

535 signals:1) a high % serines; 2) a lack of negative amino acids in the first 12 residues; 3) an aliphatic

536 amino acid at position 3 or 4.

537 [a] Although the main hit of OG0004463 (HopAG1) was a NUDIX domain, a further domain with

538 homology to ART was predicted at a different region of the protein.

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551 **Figure legends**

552 **Figure 1. Predicted NADases as type III effectors in *P. syringae* species complex.**

553 A) Enzymes that can cleave NAD$^+$. The NUDIX (nucleoside diphosphate linked to a

554 variable moiety X) enzymes and phosphodiesterases (PDEs) cleave the

555 pyrophosphate bond. ADP-ribosyltransferases (ART), nucleoside hydrolases

556 (NH), ADPR cyclases, and sirtuins cleave the N-glycosidic bond.

557 B) Predicted NADase type III effectors (T3Es) from the pangenome of *P. syringae*.

558 Eight known T3Es and five new candidates are mapped onto the maximum-

559 likelihood core genome phylogeny of *P. syringae* species complex. Strains in the

560 primary phylogroups are coloured in red in the phylogenetic tree. Ring 1-6 each

561 represents one of the six enzymatic families with NADase activity. Known T3Es

562 with ART domains are in the order (from inside to outside) of HopF/BB, HopO1,

563 AvrRpm, HopS, HopU1, HopAG1. Grey squares indicate the orthogroup of a

564 specific enzymatic domain is present in the strain but does not have a putative *hrp*

565 box in the promoter.

566

567 **Figure 2. OG18056 is an active NADase that produces a variant of cADPR.**

568 A) Alignment of a structural model of OG18056 (predicted by AlphaFold2, in cyan)

569 with a human ADPR cyclase CD38 (6VUA, in grey) and the TIR domain of human

570 SARM1 (6O0R, 561-700 aa, in light brown). For visualisation purposes, sequences

571 outside the alignment region were removed and those that did not align within the

572 region are more transparent. The position of the predicted catalytic Glu residues,

573 which are aligned in the three proteins, is highlighted with a box.

574 B) A close-up of alignments of OG18056 with TIR-hSARM1 (upper) and hCD38

575 (lower) at the predicted catalytic site showing a Glu E305 in OG18056 that is

576 aligned with the catalytic residues E642 in TIR-hSARM1 and E226 in hCD38.

577 C) Electrostatic image of the surface of the structure model of OG18056 with regions

578 of positive, neutral, and negative charge coloured in blue, white, and red

579 respectively. A molecule of NAD$^+$ was docked into the predicted catalytic pocket.

580 D) HPLC chromatograms showing the cADPR product produced by OG18056 using

581 a semi-*in vitro* assay. OG18056 and OG18056^{E305A} were expressed in *N.*

582 *benthamiana*. After immuno-precipitation, the proteins were incubated with NAD$^+$

583 for one hour before subjected to LCMS analysis. The controls were NAD$^+$ only

584 without addition of beads and immunoprecipitation of proteins from leaves

585   infiltrated with *Agrobacterium* carrying an empty vector (EV). Peaks corresponding

586   to m/z 542.06 (cADPR) and m/z 664.11 (NAD$^+$) were identified.

587   E) Immunoblots detecting the expression of OG18056 and OG18056^{E305A} in *N.*

588   *benthamiana* (input) and after immunoprecipitated (IP). The IPed samples were

589   used for the LCMS assays. OG18056 and OG18056^{E305A} were tagged with C-

590   terminal 3xFLAG. Empty vector (EV) was included as a control. Ponceau Staining

591   (PS) of the membrane was used as a loading control.

592

Figure 3: OG18056 (HopBY) is a true type III effector that produces 2'cADPR

during bacterial infection.

595   A) Immunoblots showing the induction and secretion of OG18056 in a T3S-dependent

596   manner by *P. syringae*. OG18056-HA was introduced in PstDC3000 or the T3S

597   mutant Δ*hrcC* under its native promoter. The bacteria were cultured in the rich

598   medium King's B (KB) or a Hrp-inducing minimal medium (HM). Proteins were

599   extracted from cell pellet (cp) or supernatant (sn) and OG18056 was detected by

600   western blotting using an anti-HA antibody. Ponceau Staining (PS) of the

601   membrane was used as a loading control. OG18056 is hereafter referred to as

602   HopBY.

603   B) HPLC chromatograms of *A. thaliana* leaf extracts using tissues 18 hours post-

604   inoculation with the *P. syringae* "effectorless" mutant D36E expressing HopBY.

605   Leaves treated with 10mM MgCl$_2$ was used as the control. Leaves inoculated with

606   D36E carrying an empty vector (EV), or expressing HopBY^{E305A}, HopAM1 or

607   HopAM1^{E191A} were included for comparison. All effectors were cloned under their

608   native promoter. Peaks corresponding to m/z 542.06 (cADPR) and m/z 664.11

609   (NAD$^+$) were identified.

610   C) HPLC chromatograms of different cADPR variants compared to the product of

611   HopBY (extracted from *A. thaliana* inoculated with D36E expressing HopBY).

612   2'cADPR was detected from cell lysate of *E. coli* expressing AbTIR, and 3'cADPR

613   was detected from *A. thaliana* leaves inoculated with D36E expressing HopAM1.

614   cADPR and NAD$^+$ were purchased from Sigma.

615   D) HPLC chromatogram of a mixture of samples as described in Panel C. Only one

616   peak appeared at a retention time 3.0 min, indicating that the HopBY product is

617   likely 2'cADPR. At: *Arabidopsis thaliana*; Ec: *E. coli*

Figure 4: HopBY enhanced bacterial growth and triggered disease-like symptoms in *A. thaliana*.

A) Disease symptom development in *A. thaliana* inoculated by the *P. syringae* mutant D36E carrying the empty vector (EV), or expressing HopBY, HopBY[E305A] or AvrRps4. Adult leaves of wildtype (WT) and *eds1-2* mutant were inoculated with bacterial suspensions of 5 x 10⁶ CFU/mL and symptoms were imaged at 3 days post inoculation.

B) Cell death-like phenotype induced by HopBY at a high inoculum dose (5 x 10⁸ CFU/mL) in an EDS1-independent manner. D36E expressing individual effectors were used to inoculate *A. thaliana* adult leaves, which were imaged one day post inoculation.

C) Bacterial populations (in Log CFU/cm²) measured at 0 and 3 days post inoculation in wildtype *A. thaliana*. D36E transformants were used to inoculate adult leaves at 5 x10⁵ CFU/mL. Individual data points with different colors represent results from three separate experiments. Tukey-HSD significance groups extracted from ANOVA are presented ($p < 0.05$). HopBY was cloned in the vector pUCP20tk but AvrRps4 was cloned in the vector pBBR1MCS-5. Therefore both empty vectors (pUCP EV and pBBR EV) were included as controls.

Fig 5: HopBY homologs are present in diverse bacterial species as type III or type VI effectors.

A) Maximum-likelihood phylogeny of HopBY homologs using structure-guided protein sequence alignment. The homologs are grouped in two clades with the *P. syringae* HopBY labeled as "1". Tips are labelled as green (T3Es with a *hrp* box in the promoter) or pink (putative T6Es). The predicted Hrp2 T3E was labelled as black. The scale shows substitutions per site.

B) AlphaFold2 models of HopBY homologs, which show diverse overall structures. Numbers correspond to panel A. 1: *P. syringae coronafaciens* ICMP 4457, 2: *Brenneria* sp. EniD312, 3: *Gammaproteobacteria* RS470, 4: *Paraburkholderia* sp. ZP32-5, 5: *Duganella* sp. CF517, 6: *Pseudomonas frederiksbergensis* PgKB32. The HopBY-like ADPR cyclase domain is always on the C terminus of each protein, which is highlighted in cyan.

651   **C)** Sequence logo showing the conservation of residues in the region surrounding the

652   catalytic Glu residue (corresponding to positions 300-310 aa in the *P. syringae*

653   HopBY). W301 and E305 are 100% conserved in all the 44 homologs.

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781
782

783

A

OG18056 E305
hCD38 E226
hSARM1 E642

B

E642
E305

E305
E226

C

D

| Control | EV | OG18056 | OG18056 E305A |

Intensity (counts x10⁴)

cADPR
NAD⁺

RT(mins)

E

kDa	EV	OG18056	OG18056^E305A	
40-				INPUT α-FLAG
				PS
40-				IP α-FLAG

784

785

786

A

⑤ *Duganella* spp.

③ *Gammaproteobacteria*

Pseudomonas spp.

① **P. syringae**

② *Brenneria* spp.

Pectobacterium colocasium

Pectobacterium spp.

④ *Paraburkholderia sp.*

Rhodanobacter sp.
Betaproteobacteria sp.

⑥ *Pseudomonas* spp.

Pseudomonas migulae

0.3

● T3 effector with *hrp* box
● Putative T6 effector
● T3 effector with PIP box

B

① ② ③ ④ ⑤ ⑥

C

787