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**Author for correspondence:**
G. M. Preston
e-mail: gail.preston@plants.ox.ac.uk

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# Local adaptation is associated with zinc tolerance in *Pseudomonas* endophytes of the metal-hyperaccumulator plant *Noccaea caerulescens*

H. N. Fones[1], H. McCurrach[2], A. Mithani[3], J. A. C. Smith[2] and G. M. Preston[2]

[1]Biosciences, University of Exeter, Stocker Road, Exeter EX4 4QD, UK
[2]Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
[3]Department of Biology, Syed Babar Ali School of Science and Engineering, Lahore University of Management Sciences (LUMS), DHA, Lahore 54792, Pakistan

GMP, 0000-0003-3882-4438

Metal-hyperaccumulating plants, which are hypothesized to use metals for defence against pests and pathogens, provide a unique context in which to study plant–pathogen coevolution. Previously, we demonstrated that the high concentrations of zinc found in leaves of the hyperaccumulator *Noccaea caerulescens* provide protection against bacterial pathogens, with a potential trade-off between metal-based and pathogen-induced defences. We speculated that an evolutionary arms race between zinc-based defences in *N. caerulescens* and zinc tolerance in pathogens might have driven the development of the hyperaccumulation phenotype. Here, we investigate the possibility of local adaptation by bacteria to the zinc-rich environment of *N. caerulescens* leaves and show that leaves sampled from the contaminated surroundings of a former mine site harboured endophytes with greater zinc tolerance than those within plants of an artificially created hyperaccumulating population. Experimental manipulation of zinc concentrations in plants of this artificial population influenced the zinc tolerance of recovered endophytes. In laboratory experiments, only endophytic bacteria isolated from plants of the natural population were able to grow to high population densities in any *N. caerulescens* plants. These findings suggest that long-term coexistence with zinc-hyperaccumulating plants leads to local adaptation by endophytic bacteria to the environment within their leaves.

## 1. Introduction

Metal-hyperaccumulating plants maintain exceptionally high concentrations of metals in their aerial tissues [1–4], often exceeding 1% of tissue dry biomass for elements such as zinc, cadmium, nickel and manganese, a phenotype that is thought to protect them from herbivory [5–12] and disease [13–15]. There is evidence that the use of metals in defence has led to an evolutionary trade-off between metal hyperaccumulation and some pathogen-induced defences [16–18]. At present, however, little evidence is available concerning the effect of metal hyperaccumulation and metal-based defences upon the endophytic bacteria of hyperaccumulator plants. Two studies have surveyed the endophytes associated with nickel hyperaccumulator plants on natural serpentine soils [19,20]. Both reported that these bacteria showed high nickel tolerance, supporting the notion that the plants provide a high-metal environment for bacteria. Bacterial nickel tolerance was found to vary in proportion to nickel concentrations in different tissues, while bacterial population densities decreased as nickel concentrations increased [19]. Similarly, high cadmium tolerance was found for the bacterial endophytes of a cadmium hyperaccumulator growing on mine tailings [21]. Thus, high metal tolerance is associated with bacterial growth within metal-hyperaccumulating plants.

In previous work, we demonstrated that zinc concentrations in the aerial tissues of plants of a natural *Noccaea caerulescens* (synonym *Thlaspi caerulescens*) population growing on an abandoned lead–zinc mine at Hafna, Snowdonia, UK, [22] are higher than those found to prevent the growth of pathogenic *Pseudomonas syringae* bacteria under laboratory conditions [15], suggesting that zinc tolerance is important for naturally occurring endophytes of these plants. Here, we investigate the possibility that the endophytic pseudomonads of metal hyperaccumulators have developed increased metal tolerance as a result of local adaptation to the metal-rich environment of the hyperaccumulator leaves, a scenario that might drive the evolution of further metal hyperaccumulation in plants that use metals as a defence against pathogenic microorganisms.

In this study, endophytic *Pseudomonas* associated with a natural population of the zinc hyperaccumulator *N. caerulescens* were characterized and compared with those associated with an artificial field population. Bacterial zinc tolerance and virulence on *N. caerulescens* were determined and the distribution of these traits across a phylogeny of the isolated bacteria was examined. The results provide evidence that bacteria isolated from the natural population of *N. caerulescens* are better able to grow and cause disease in this metal hyperaccumulator. These findings support the idea of local adaptation by endophytic bacteria communities in an established, natural population of *N. caerulescens*, and strengthen the concept of plant pathogens as a selective force in the evolution of metal hyperaccumulation.

## 2. Material and methods

### (a) Field sites

Plants of *N. caerulescens* (J.Presl & C.Presl) F.K.Mey. (=*Thlaspi caerulescens* J.Presl & C.Presl) were studied at two field locations: an artificial population cultivated at Wytham, Oxfordshire, UK (51°47′ N, 1°39′ W) and a natural population growing on wasteland at Hafna mine, an abandoned lead–zinc mine in Snowdonia National Park, Conwy, UK (53°07′ N, 3°49′ W [22]). The artificial population was generated from seed collected from *N. caerulescens* at Prayon, Belgium (50°35′ N, 5°40′ E [23]). Plants were precultured in a glasshouse for eight weeks on soil supplemented with zinc oxide to contain either 0, 1, 2 or 5 g $kg^{-1}$ Zn (w/w). They were then transferred to two closely adjacent field sites at Wytham. At each site, plants grown on different zinc treatments were split equally between three randomized blocks.

### (b) Measurement of leaf zinc concentrations

Leaf material was sampled from plants of *N. caerulescens* at all field sites and pools of 10–30 plants per treatment created. Zinc content in pooled samples of leaf biomass was measured following oven drying and extraction in concentrated nitric acid by atomic absorption spectrophotometry as described [24].

### (c) Endophyte collection, identification and *in planta* growth assays

*Noccaea caerulescens* leaves were collected at both field sites, sealed into plastic bags and transferred to the laboratory for further analysis. From the artificial population at Wytham, 10 leaves per plant per zinc treatment were sampled. From the natural population at Hafna mine, 10 leaves per plant were sampled. Incidence of disease symptoms in sampled plants was recorded. Leaves were surface sterilized by successive immersion in 10% (v/v) sodium hypochlorite solution and 100% ethanol for 5 min each. The sterilized leaves were rinsed in sterile, distilled water and dried. Each leaf was then macerated and incubated at room temperature for 5 min in 1 ml of potassium phosphate buffer at pH 6.8. One hundred microlitre aliquots of this buffer were spread onto King's B (KB) agar, which is particularly conducive to the growth of *Pseudomonas* species [25]. Plates were incubated at 28°C for 48 h. Colonies were picked into KB broth and incubated overnight. Cultures were supplemented with 50% (v/v) glycerol to a final concentration of 20% (v/v) glycerol and stored at −80°C. All endophyte strains were subjected to LOPAT and GATTa assays [26]. In addition, *16S*, *rpoD* and *gyrB* genes were sequenced (primers are given in electronic supplementary material, table S1) and used as queries for NCBI BLAST. *In planta* growth and pathogenicity assays were carried out as described [15].

### (d) Measurements of bacterial zinc tolerance

All bacterial strains isolated from *N. caerulescens* plants were grown in KB broth supplemented with a range of zinc concentrations from 0 to 20 mM, and the increase in $OD_{600}$ of the medium over 24 h recorded. Bacterial growth response curves for zinc were then created and used to determine the concentration of zinc causing a 50% reduction in bacterial growth ($IC_{50}$; [27]). Growth response curves were also clustered by shape, using *k*-means clustering in 'R' [28], into groups or classes representing different patterns of response to zinc. *k*-means clustering maps data to the nearest mean value, where '*k*' is the number of means; here, $k = 4$ was selected as this gave clear, discrete clusters. Strains in group 4 were the most tolerant, able to grow at 15 mM Zn, whereas those in group 1 showed strongly reduced growth even at 5 mM Zn. Both measures of zinc tolerance were used to describe the strains.

### (e) Phylogeny reconstruction

Sequences of endophyte *rpoD* genes were used to build a phylogenetic tree. Alignment was carried out using MUSCLE (EBI [29,30]). Reference strain sequences were chosen based on BLAST results for the endophytic bacteria and obtained from NCBI (www.ncbi.nlm.nih.gov). GenBank IDs are given in electronic supplementary material, table S2. Following the initial alignment, strains with poor alignment to the rest or with poor sequence quality were removed from the analysis. Remaining sequences were trimmed to the region which consistently aligned well across all sequences, and the alignment repeated. Resulting good-quality alignments were used to produce a single gene tree. *rpoD* gene sequences were obtained from 60% (144/244) of isolated strains and 60% (86/144) of these sequences were of sufficient quality and sufficiently well aligned to use in phylogeny reconstruction. Phylogeny reconstruction was performed in MEGA [31] by maximum parsimony [32], using the Close-Neighbour-Interchange algorithm [33] with search level 3 in which the initial trees were obtained with the random addition of sequences with 10 replicates. An estimate of the trees' robustness was obtained by conducting a bootstrap analysis [34] with 500 replicates.

## 3. Results

### (a) Zinc hyperaccumulation is negatively correlated with disease incidence in a natural population of *Noccaea caerulescens*

To determine whether an established population of zinc-hyperaccumulating plants would host a population of locally

adapted pathogens able to cause disease in high-zinc conditions, we compared zinc concentration and disease incidence in *N. caerulescens* plants of the natural population at Hafna Mine, Snowdonia, to *N. caerulescens* plants established in an artificial population at two field sites at Wytham. *N. caerulescens* plants at Wytham showed significantly increased leaf zinc concentration when grown on increasingly high-zinc soil, at both sites (figure 1*a*,*b*) (ANOVAs: Site 1, $p < 0.0005$; Site 2, $p = 0.009$). Plants from the natural population displayed high concentrations of zinc in their aerial tissues, above the threshold of 1.0% of tissue dry biomass regarded as defining zinc hyperaccumulation [35]. There were two spatially separate patches of *N. caerulescens* at the Hafna Mine site. Interestingly, the plants in these two sub-populations contained significantly different concentrations of zinc (ANOVA, $p = 0.0001$) (figure 1*c*), with the sub-population that showed higher zinc concentrations consisting of plants that appeared healthy (figure 1*d*) while the other sub-population contained plants displaying disease symptoms (figure 1*e*). No plants of the artificial field population in Wytham displayed symptoms of disease at any time.

## (b) Plants of the natural population of *Noccaea caerulescens*, and plants with higher zinc concentrations, hosted endophytes with greater zinc tolerance

To investigate whether the leaves of zinc-hyperaccumulating *N. caerulescens* provide a local environment that imposes a selective pressure in favour of zinc tolerance, we measured and compared the zinc tolerance of bacteria isolated from plants of the artificial and natural populations. Zinc tolerance curves showing bacterial growth versus zinc concentration were produced for each endophyte strain, which were used to determine $IC_{50}$ values for zinc for each strain. The endophytes isolated from leaves from the artificial population had a greater range of zinc tolerances by this measure, but the median for the strains isolated from the natural population was significantly higher than that for the artificial population ($IC_{50}$ of 9.5 versus 7.0 mM, respectively; Mann–Whitney test: $p < 0.0005$). Zinc tolerance curves for individual strains were clustered by shape using *k*-means clustering. These clusters then served to define four tolerance groups, with group 1 being the least, and group 4 the most, tolerant strains (electronic supplementary material, figure S1).

The median tolerance group of the endophytes increased as the plant zinc concentration increased, either with zinc treatment in the artificial population or between the two patches of plants in Snowdonia (figure 1*f*). Kruskal–Wallis tests on these data produce significant *H*-statistics (Wytham zinc treatments, $p = 0.014$; Snowdonia sub-populations, $p < 0.0001$). In conjunction with the results shown in figure 1*a*–*c*, this indicates that greater zinc concentrations within the plants were correlated with higher zinc tolerance among their endophytes.

## (c) Artificial and natural populations of *Noccaea caerulescens* supported different bacterial endophytes

The majority of isolated strains from both artificial and natural populations belonged to the genus *Pseudomonas*, with some representatives of *Xanthomonas*, *Erwinia*, *Enterobacter*, *Pantoea*, *Escherichia* and *Aeromonas*. Pseudomonads were of particular interest, as previous work has shown that some *Pseudomonas* species can be pathogenic on *N. caerulescens* [15]. By focusing on *Pseudomonas*, direct comparison of the results obtained here to those in our previous study was facilitated [15]. KB agar was therefore selected as an isolation medium [25]. Since KB is biased in favour of pseudomonads, we limit further consideration to this genus. The composition of the two populations was widely different, with Wytham plants supporting mainly strains whose top BLAST hit was to *P. aeruginosa*, whereas Snowdonia plants supported mainly strains whose top BLAST hit was to *P. syringae*, *P. graminis* and *P. fluorescens* (electronic supplementary material, figure S2).

## (d) Phylogenies of *Pseudomonas* endophyte strains from natural and artificial field populations of *Noccaea caerulescens* suggest multiple origins of zinc tolerance

To investigate whether zinc-tolerant strains of endophytes were related, an *rpoD* gene tree was created for all endophytic pseudomonads for which high-quality *rpoD* gene sequences could be obtained. Figure 2 shows the resulting tree with both measures of zinc tolerance ($IC_{50}$ and tolerance group) indicated. Highly zinc-tolerant strains are distributed across the tree and are not confined to strains isolated from either population of *N. caerulescens*. Also included are reference strains from the genus *Pseudomonas*, and non-*Pseudomonas* reference strains *Hahella chejuensis*, *Azotobacter vinelandii* and *Acinetobacter* ADP1. These show this tree to be in agreement with phylogenies produced by other researchers for *Pseudomonas* [36], with strains of Cluster I separated from Cluster II, and, within the clusters, the *aeruginosa*, *syringae* and *fluorescens* complexes differentiated. These deep branches in the tree also have high bootstrap support (99%, 99% and 97%, respectively; figure 2). The average $IC_{50}$ for zinc in these three species complexes were similar (9.8 mM, 9.5 mM and 9.0 mM, respectively), and species complex was not a significant predictor of $IC_{50}$ (ANOVA, $p = 0.52$). On the other hand, tolerance groups were not uniformly distributed between the three species complexes, with more group 4 strains in the *syringae* and *fluorescens* complexes, and more group 2 strains in the *aeruginosa* complex ($\chi^2$-goodness-of-fit test to uniform distribution of groups; $p < 0.001$). Smaller monophyletic clades with high bootstrap support can be seen within the *syringae* and *fluorescens* clusters, some of which contain strains with similar zinc tolerances, while others include a range of zinc $IC_{50}$ values and tolerance groups.

## (e) Artificial and natural populations of *Noccaea caerulescens* supported different bacterial endophytes

To identify the strains isolated, we sequenced *16S*, *rpoD* and *gyrB* genes and used the results to query the NCBI database using BLAST [37] (electronic supplementary material, table S3). Notably, a higher incidence of strains closely related to *P. syringae* was found in Snowdonia (electronic supplementary material, figure S2), which is consistent with disease incidence at this site, since at least some strains of *P. syringae*

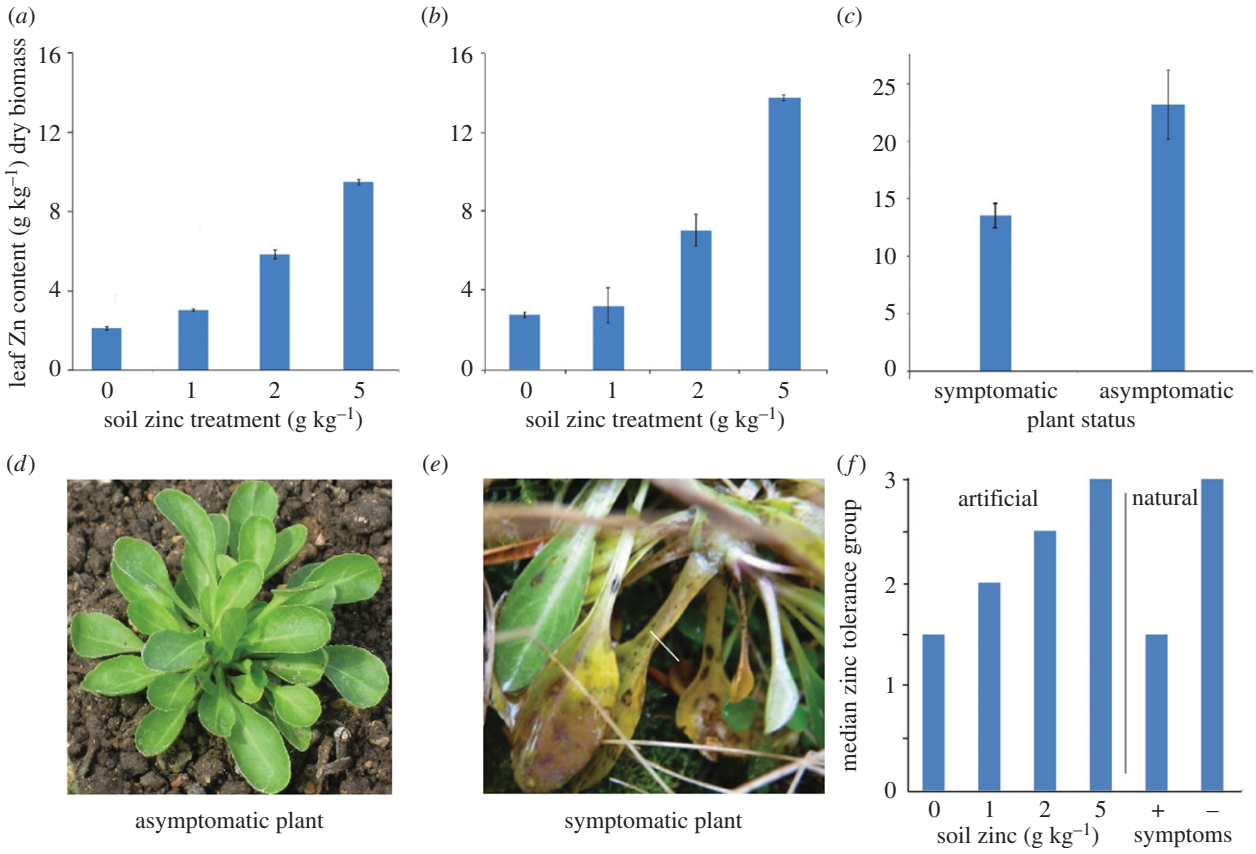


**Figure 1.** Zinc accumulation, tolerance and disease incidence in natural and artificial *Nocaea caerulescens* populations. Zinc concentration in leaves of plants of the artificial population (Prayon genotype) grown at two distinct field sites at Wytham, UK, was measured at the end of the experimental period (*a*,*b*). Values shown are means ± s.e. ($n = 3$ samples of 10–30 pooled plants; at least three technical replicates performed per sample). Two spatially distinct sub-populations of *N. caerulescens* were distinguished in the natural population at Hafna Mine, Snowdonia; two leaves per plant were sampled from randomly selected plants from each patch and zinc concentrations measured (*c*); these two sub-populations were designated 'asymptomatic' and 'symptomatic' (*d*,*e*, respectively), as disease was observed in one only. Strains from both the artificial and natural populations were grown on KB medium supplemented with a range of zinc concentrations from 0 to 20 mM, and the increase in $OD_{600}$ of the medium over 24 h recorded. Bacterial growth curves were then clustered using *k*-means clustering into 'tolerance group' representing different patterns of response to zinc (see electronic supplementary material, figure S1). Median zinc tolerance group (1–4, where 1 = least tolerant and 4 = most tolerant) of endophytic bacteria isolated from plants of the artificial population of *N. caerulescens* grown at Wytham, on four different zinc soil treatments is shown (*f*), as is median zinc tolerance group of endophytic bacteria isolated from plants of the natural population in Snowdonia, from spatially separate patches in which symptoms were (+) or were not (−) apparent (*f*). (Online version in colour.)

are pathogenic on *N. caerulescens* [15]. Endophytes identified as *P. syringae* and *P. graminis* also caused symptoms in *N. caerulescens* on reinoculation (electronic supplementary material, table S4). Further details about the identity of different bacterial strains at the two sites and about reinoculation experiments are given in the electronic supplementary material.

### (f) On inoculation into *Noccaea caerulescens* plants grown on low zinc, symptoms were observed with *Pseudomonas syringae* and *Pseudomonas graminis* strains

For further insight into whether any of the strains isolated from the Snowdonia plants could be responsible for observed disease symptoms, all strains from these plants were inoculated into *N. caerulescens* plants (from Prayon, Belgium, as used for the artificial population) grown on low zinc in the laboratory. Plants were assessed at 24 h, and those showing rapid necrosis were excluded from further analysis due to possible onset of a non-host response. Symptom development in the remainder was recorded over 72 h. Of the 83 strains isolated, 14 induced symptoms (electronic supplementary material, table S4). All these had at least one top BLAST hit to *Pseudomonas*; in 10 cases, there was a top BLAST hit to *P. syringae* or *P. graminis* (electronic supplementary material, table S3). Considering the known role of many *P. syringae* strains as causative agents of disease (e.g. [15]), these species are plausible candidates to be the causal agents of the observed disease symptoms.

### (g) Pathogenicity of endophytes on *Noccaea caerulescens* depends on both zinc tolerance and association with the natural population of *Noccaea caerulescens*

To determine the effect of zinc tolerance on the virulence of candidate pathogens, six endophyte strains belonging to the species *P. syringae* (determined using sequence data and LOPAT and GATTa assays) and the previously studied laboratory strain *P. syringae* pv. maculicola M4 were inoculated into *N. caerulescens* plants (from Prayon, Belgium) grown with 0.04, 10, 30 or 300 µM zinc. Four strains were isolated from the natural *N. caerulescens* population, and displayed a range of zinc tolerances; the remaining two were from the artificial *N. caerulescens* population and displayed high-zinc

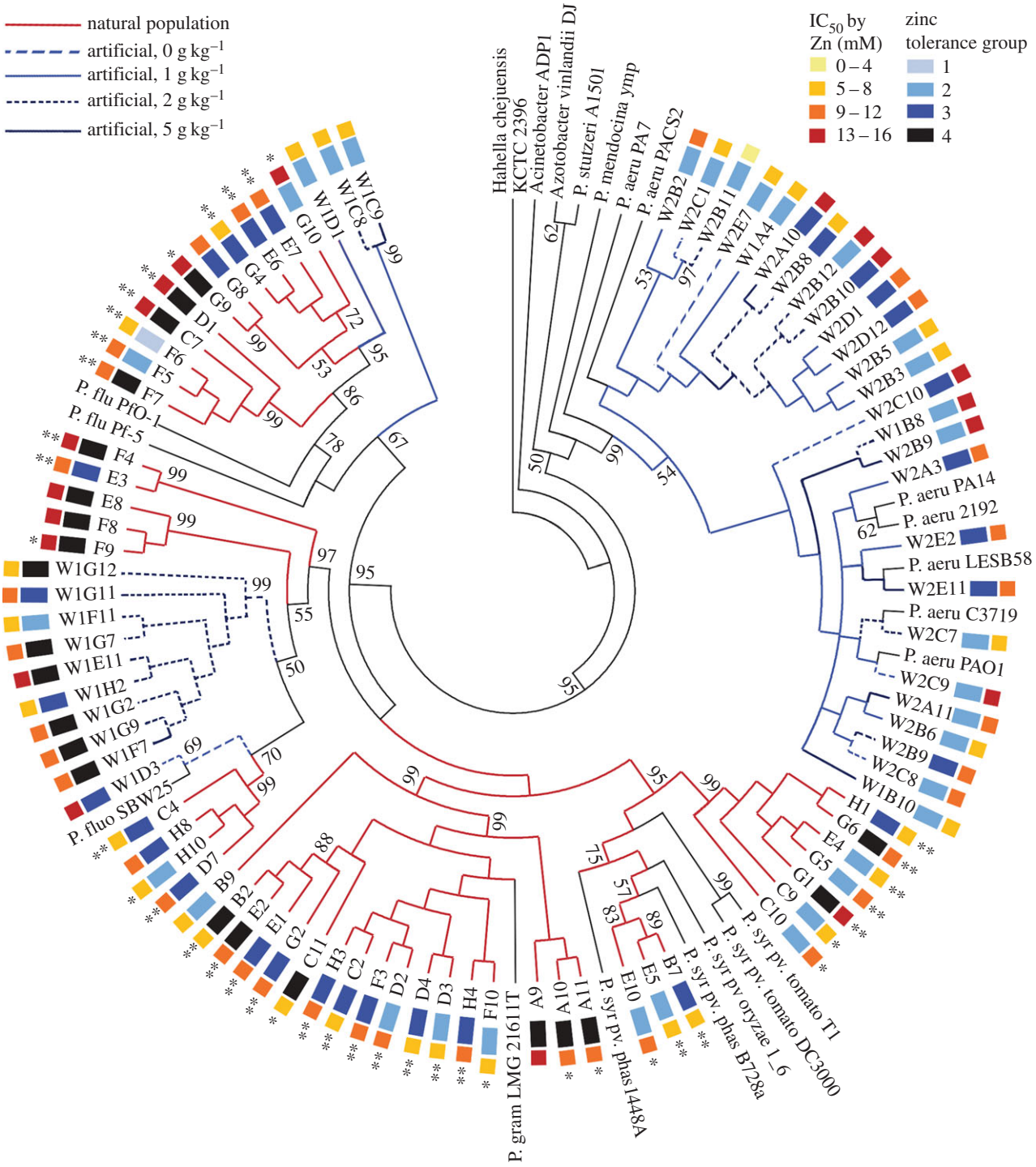


**Figure 2.** *rpoD* gene tree of Wytham and Snowdonia strains. The bootstrap consensus tree from 120 most parsimonious trees is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to those branches where the bootstrap value exceeds 50% [27]. Branches are coloured red for endophytes of plants from the natural population in Snowdonia and blue for endophytes of plants from the artificial population at Wytham. Within the natural population, ** indicates that the plant from which the endophyte was isolated was symptomatic, whereas * indicates that the plant was from the same spatial sub-population as the symptomatic plants; no asterisk indicates that the isolate was from the asymptomatic sub-population. Within the artificial population, blue branches are further colour-coded to indicate the zinc treatment applied to the plant from which the endophyte was extracted (light blue: dashed: no added zinc, solid: 1 g zinc added per kilogram of soil; dark blue: dashed 2 g $kg^{-1}$, solid: 5 g $kg^{-1}$). Zinc tolerance groups based on groupings for all strains on KB are shown in black and shades of blue, with group 4 (highest) in black, through to group 1 (lowest) in pale blue. Zinc concentrations giving $IC_{50}$ for strains are indicated with a heat map where red is highest (13–16 mM Zn) and yellow lowest (0–4 mM).

tolerance (table 1). As previously reported [15], *Psm* was able to multiply within plants grown on 0.04 and 10 µM Zn, but showed reduced or no growth in plants grown on 30 µM or 300 µM Zn.

Bacterial growth *in planta* is shown in figure 3. The four strains isolated from the natural population of *N. caerulescens* were all able to grow *in planta* when the plants were grown on 0.04 µM Zn. The ability of these strains to multiply in plants grown on higher concentrations of zinc was correlated with their zinc tolerance, with the most tolerant, strain SnC10 ($IC_{50}$ for Zn = 11.8 mM) able to multiply in all plants, although with reduced growth at higher zinc; strains SnB11 and SnD3 ($IC_{50}$ = 7.1 and 7.6mM, respectively) able to multiply only in plants grown on 30 µM Zn or lower, again with

**Table 1.** Zinc tolerance of strains selected for *in planta* growth assays, showing zinc tolerance groups as defined by *k*-means clustering (electronic supplementary material, figure S1) and $IC_{50}$ values for zinc.

| strain | zinc tolerance group | $IC_{50}$ [Zn] (mM) |
|---|---|---|
| W1D7 | 2 | 10.0 |
| W1E11 | 1 | 11.3 |
| SnD3 | 3 | 7.63 |
| SnD4 | 3 | 6.75 |
| SnC10 | 3 | 11.8 |
| SnB11 | 3 | 7.06 |
| *Psm* | not tested | 7.25 |

less success at higher zinc; and the least tolerant strain, SnD4 ($IC_{50} = 6.7$ mM), only able to multiply in the plants grown on 0.04 µM Zn. *Psm* has an $IC_{50}$ of 7.25 mM, placing it between SnB11 and SnD3, whose *in planta* behaviour it closely replicated. By contrast, neither of the strains isolated from the artificial population of *N. caerulescens* was able to multiply *in planta*, regardless of plant zinc treatment, despite the high *in vitro* zinc tolerances of these two bacterial strains ($IC_{50} = 10$ and 11.3 mM).

## 4. Discussion

In this work, we investigated the possibility that populations of bacterial endophytes of metal-hyperaccumulating plants become locally adapted to the metal-rich environment they encounter within these plants. Previously, we have demonstrated that endophytic bacteria of the zinc hyperaccumulator *N. caerulescens* show higher zinc tolerance when compared with bacteria pathogenic on related non-accumulator plants [15], an idea supported by other studies of bacteria associated with metal-hyperaccumulating plants [18,19,38–40]. Considered alongside evidence for a role of hyperaccumulated metals in defence of plants such as *N. caerulescens* against disease [13–15,41,42], this suggests that local adaptation of pathogens to high-metal concentrations in the rhizosphere and phyllosphere of metal-accumulating plants might drive the evolution of further defensive hyperaccumulation in a form of coevolutionary arms race [15,43].

Here, we have shown that bacteria isolated from the zinc-hyperaccumulating plant *N. caerulescens* display a degree of zinc tolerance correlated with the zinc concentrations found in the leaves of the plants from which they were isolated. This result supports the idea that the leaf environment provided by this zinc hyperaccumulator exerts a selection pressure for zinc tolerance. While in theory it is possible that disease leads to reduced metal accumulation, any reduction in metal uptake capacity would occur as a result of disease symptoms; zinc accumulation, however, occurs throughout the life of the plant, so that any measureable differences in the zinc content of aerial tissues are likely to predate the infection. Additionally, this is demonstrated in laboratory experiments (e.g. [15]) in which plants were first cultivated, in a healthy state, on a range of Zn concentrations and then artificially inoculated with *P. syringae*, resulting in symptoms with severity inversely proportional to measured plant Zn content. On the other hand, there is now an extensive body of evidence indicating that cross-talk occurs between metal and biotic stress signalling at the level of ROS (e.g. [15]), hormones (e.g. [44]), transcriptomic and proteomic changes (including enzymes of the secondary metabolism, PR proteins and more, see [45] for review). This cross-talk may confer cross-protection against disease when plants are exposed to and accumulate heavy metals such as zinc. Additionally, there is mounting evidence (e.g. [6,7,15,16]) that metal hyperaccumulation does indeed protect these plants from disease, as would be expected under the hypothesis that an evolutionary arms race taking place in the zinc-rich environment of hyperaccumulators and their surroundings is responsible for driving the evolution of the metal accumulation trait.

We have also compared the zinc tolerance of bacteria isolated from a natural population (Snowdonia) of *N. caerulescens* plants to those isolated from an artificial population (Wytham) created for this study. The preponderance of more zinc-tolerant bacteria originating from the Snowdonia site, and of less zinc-tolerant bacteria from the Wytham site, suggests that the natural population of *N. caerulescens* at Snowdonia provided a selection pressure acting in favour of zinc-tolerant bacteria. The scattered distribution of zinc tolerance across the *rpoD* gene tree implies multiple origins of zinc tolerance, within and among groups of related bacteria, suggesting that this trait might be acquired relatively easily, as seen in a library of transposon mutants of *P. syringae* pv. maculicola [15]. This supports the hypothesis that, if metal accumulation evolved as a defence, rapidly evolving pathogens are likely to have driven a subsequent arms race leading to the progressive enhancement of the metal-accumulation phenotype [16].

It is possible that differences in plant genotype between the *N. caerulescens* plants in the natural and artificial environments may have influenced the establishment of endophytic bacteria. However, differences in endophyte zinc tolerance that covary with plant zinc content are seen at the within-genotype level. Thus, the findings shown in figure 1*f*, that the median zinc tolerance group into which isolated endophytes fall is positively correlated with plant zinc content in the natural population, and with the zinc treatment on which the plants were grown in the artificial population, indicate that the plant zinc content affects the zinc tolerance of its bacterial endophytes in a manner independent of plant genotype.

Interestingly, while $IC_{50}$ values for zinc are not predicted by the species complexes into which a strain falls on the *rpo*D tree, zinc-tolerance group percentages do vary between these phylogenetic groups. Strains in the *aeruginosa* complex are exclusively found in tolerance groups 2 and 3, both of which show fairly linear zinc dose–response curves (electronic supplementary material, figure S1). Strains in the *syringae* and *fluorescens* complexes are, respectively, equally or more likely to fall into zinc tolerance group 4 than groups 2 and 3. Group 4 strains show little or no growth reduction until zinc concentrations are fairly high (5–10 mM zinc; electronic supplementary material, figure S1). The ability to withstand up to 10 mM zinc without growth reduction might give these bacteria an advantage when growing endophytically in *N. caerulescens* plants exposed to high zinc.

Notably, there are only three monophyletic groups with high bootstrap support (99%) containing exclusively highly zinc-tolerant strains ($IC_{50} > 9$ mM Zn and tolerance group 3 or 4) isolated from plants of the natural population. These

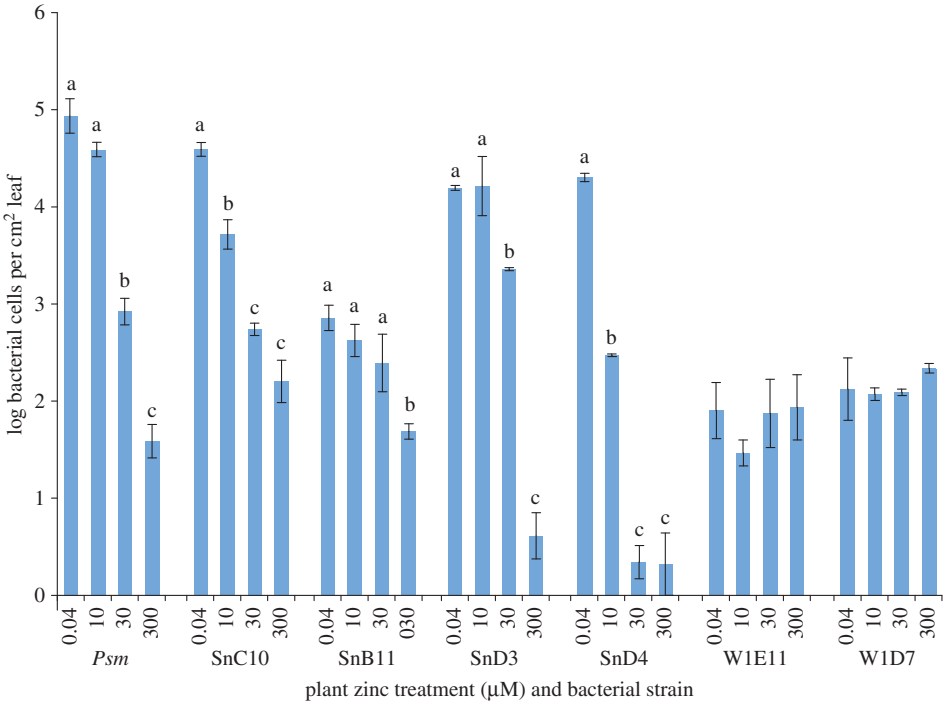


**Figure 3.** Growth of selected bacterial strains isolated from leaves of *N. caerulescens* plants of natural (SnC10, SnB11, SnD3, SnD4) or artificial populations (W1E11, W1D7). Plants were grown on 0.04, 10, 30 or 300 μM Zn and bacteria were inoculated into 10-week-old plants at $10^6$ cfu $ml^{-1}$ in 10 mM $MgCl_2$. *Pseudomonas syringae* pv. maculicola is included for comparison. Bacterial cell counts were estimated by plating three independent samples of homogenized leaf tissue onto KB-CFC agar after 0 and 5 days. Representative day 5 data from one of two replicate experiments is shown; no differences in bacterial population were detected on day 0. Zinc was a significant predictor of bacterial numbers at 5 days post-inoculation for *Psm* and all strains isolated from the natural population, but not for strains isolated from the artificial population (GLMs; $p \leq 0.0005$, $<0.0005$, 0.011, $<0.0005$, $<0.0005$, 0.638 and 0.693, for strains from left to right across the graph). Where zinc had a significant effect, Bonferroni's simultaneous comparisons were carried out. Means that were not significantly different (within each strain) are marked with the same letter; error bars represent $\pm$ s.e. (Online version in colour.)

are very small groups (G8 & 9, F4 & E3, E8, F8 & F9), suggesting that the trait was recently acquired. Elsewhere on the tree, such strains tend to be found in clades with high bootstrap support that contain a greater variety of zinc tolerance phenotypes (for example, the highly tolerant strains G1 and G9, which are found alongside a range of less tolerant strains), again implying recent, repeated acquisitions of tolerance. Zinc-tolerant strains isolated from the artificial *N. caerulescens* population, by contrast, dominate, and are exclusively found within one clade (BS 99%) within the *fluorescens* complex.

This might indicate that the established, high-zinc environment of the natural population tends to select for the zinc tolerance trait wherever it arises, while the zinc-tolerant bacteria from the artificial site represent an earlier evolution of tolerance unrelated to the sudden, artificial appearance of *Noccaea* plants. The scattered distribution of zinc-tolerant bacteria across the tree could also reflect horizontal gene transfer among bacteria in Snowdonia, in addition to mutation towards greater zinc tolerance.

While pathogens isolated from the natural population were able to infect *N. caerulescens* under laboratory conditions, the reverse was not true of even highly zinc-tolerant bacteria from the artificial population. We have previously shown that the type 3 secretion system (T3SS), used to deliver bacterial disease effectors to the host, is essential for the growth of *P. syringae* pv. maculicola (*Psm*) *in planta* in *N. caerulescens* [15]. This indicates that, despite the importance of zinc as a defence [15] and the loss of certain other defences by the metal hyperaccumulators [16,17], the plants retain defences against which the T3SS is necessary, probably including some elements of PAMP-triggered immunity (PTI: [46,47]). It is also possible that some elements of effector-triggered immunity (ETI: [46,48]) are retained. Thus, to infect *N. caerulescens*, bacteria require both zinc tolerance and the ability to overcome pathogen-induced plant defences. As a result, *N. caerulescens* may only be vulnerable to pathogenic bacteria such as *P. syringae*, which possess the T3SS and effectors, but which have also been exposed to a zinc-rich environment and acquired zinc tolerance. We hypothesize that at the former mine site in Snowdonia, the natural population of *N. caerulescens* has selected for zinc-tolerant, *N. caerulescens*-specific pathogens. These are able to infect *N. caerulescens*, while endophytic bacteria from the artificial population, with no long-term exposure to *N. caerulescens*, proved non-pathogenic.

In conclusion, this study provides evidence for local adaptation of endophytic bacteria to the zinc-rich environment of leaves of *N. caerulescens* plants growing on a zinc-rich substrate. Pathogenicity, however, requires more than simply zinc tolerance, and was only found to be prevalent in the natural population. It will be of interest to determine which elements of PTI and ETI are retained by *N. caerulescens*, and whether these occur in all *N. caerulescens*, or vary with the degree of zinc availability, tolerance and hyperaccumulation in different populations. Investigation of endophytes and pathogens of other populations, and their ability to infect plants of other populations, would contribute to a more complete picture of the emergence and ongoing evolution of hyperaccumulation and associated endophytes.

Data accessibility. *rpoD*, *gyrB* and *16S* sequences used in BLAST searches and to reconstruct the endophyte phylogeny in figure 2 are deposited in GenBank; accession numbers KX181734 - KX181845. *rpoD* sequence

alignment and tree are deposited in TreeBase; http://purl.org/phylo/treebase/phylows/study/TB2:S18035. All other data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.qb74m.

Author contributions. H.N.F. developed and performed experiments, analysed data and wrote the manuscript; H.M. performed experiments; A.M. produced tolerance clusters; J.A.C.S. and G.M.P. supervised the work and wrote the manuscript.

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