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The genetic basis of cadmium resistance of *Burkholderia cenocepacia*

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Summary

Burkholderia **species are highly resistant to heavy metals (HMs), yet their resistance mechanisms are largely unknown. In this study we screened 5000 mini-Tn*5* transposon insertion mutants of *Burkholderia cenocepacia* H111 for loss of cadmium tolerance. Of the four genes identified three affected outer membrane biogenesis and integrity or DNA repair. The fourth gene, BCAE0587, encoded a P1-type ATPase belonging to the CadA family of HM exporters. CadA-deficient strains lost the ability to grow in the presence of cadmium, zinc and lead, whereas resistance to nickel, copper and cobalt was not affected. Expression studies using a transcriptional fusion of the *cadA* promoter to *gfp* confirmed this specificity, as induction was only observed in presence of cadmium, zinc and lead. The promoter activity was found to be highest at neutral pH with an activation threshold of 30 nM cadmium. Inoculation of the HM-hyperaccumulating plant *Arabidopsis halleri* with a RFP-marked derivative of *B. cenocepacia* H111 containing the P_{cadA}–*gfp* fusion demonstrated the applicability of this biosensor for monitoring cadmium at the single cell level in a natural environment.**

Introduction

Heavy metal (HM) contamination of soils is a worldwide environmental concern. Soil HMs are toxic for plants, animals and humans, as well as for microbes (Baath, 1989; Giller *et al*., 2009). Numerous studies have shown that the addition of HMs to soil causes a reduction of the microbial diversity and a change in the structure of the resident bacterial communities, with some genera becoming dominant over others (reviewed in Kozdroj and van Elsas, 2001). For example, it has been previously demonstrated that treatment of forest soil with cadmium strongly enriched the natural microbial community for *Burkholderia* species (Lazzaro *et al*., 2006; 2008; Schonmann *et al*., 2008).

Cadmium has been reported to be more toxic to microbes than copper, zinc and lead (Baath, 1989). Cadmium interferes with cysteine biosynthesis and binds to glutathione and the sulfhydryl groups of proteins, thereby inhibiting their function (Nies, 1999; Helbig *et al*., 2008). It has also been reported that cadmium causes DNA damage (Mitra and Bernstein, 1978; Badisa *et al*., 2007). Cadmium resistance mechanisms are diverse in bacteria and include metal exclusion via reduced membrane permeability, intra- or extracellular sequestration or active export (Bruins *et al*., 2000). Among these different possibilities active export has been suggested as the main mechanism in cadmium-tolerant bacteria. Four active export systems have been reported to confer resistance to cadmium in bacteria: CadA (Smith and Novick, 1972; Nucifora *et al*., 1989), Ncc (also exporting cobalt and nickel) (Schmidt and Schlegel, 1994), Czc (also exporting cobalt and zinc) (Nies, 1995), and the more recently discovered CzcP (Scherer and Nies, 2009). CzcP and Ncc were for a long time considered to be the major HM export systems in Gram-negative bacteria, while the P1-type ATPase CadA, which was first identified in *Staphylococcus aureus*, *Listeria* and *Lactococcus* species (Lebrun *et al*., 1994; Liu *et al*., 1997; Bal *et al*., 2003), was referred to as the 'efflux system of Gram-positive bacteria' (Silver and Phung, 1996). Only recently were export systems homologous to CadA identified in Gram-negative bacteria, e.g. in *Stenotrophomonas maltophilia*, *Pseudomonas putida* or *Escherichia coli* (Nies, 2003).

Although previous studies have shown that cadmium specifically selects for *Burkholderia* species, there is nothing known about the underlying molecular mechanism of cadmium resistance in this genus. The aim of the present study was therefore to identify the molecular determinant(s) of cadmium resistance in the model strain *Burkholderia cenocepacia* H111 (Romling *et al*., 1994). A screen of 5000 mini-Tn*5* transposon insertion mutants

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identified four genes that affected resistance to 1 mM $CdSO_4$: three of the genes encoded functions required for outer membrane biogenesis or DNA repair and thus influence the general fitness of the cell, while the fourth gene, which affected cadmium resistance most dramatically, was found to encode a CadA-family P1-type ATPase. We show that this exporter not only confers resistance to cadmium but also to high concentrations of zinc and lead and that expression of *cadA* is induced by the same three HMs. The *cadA* promoter region was used to construct a green fluorescent protein (GFP)-based cadmium biosensor, which was found to be induced when it colonized the roots of the HM-hyperaccumulating plant *Arabidopsis halleri*.

Results and discussion

Identification of genes in B. cenocepacia *required for cadmium resistance*

In order to identify the genetic determinants of cadmium resistance in *B. cenocepacia* H111, approximately 5000 random mini-Tn*5* transposon insertion mutants (Huber *et al*., 2002) were screened for lack of growth in LB medium supplemented with 1 mM $CdSO_4$. Seven of the mutants were unable to grow in medium supplemented with cadmium, while growth was unaffected in medium without $CdSO_4$. For each of these mutants, the DNA flanking the transposon insertion site was amplified by arbitrary PCR (O'Toole and Kolter, 1998), sequenced and blasted against the draft genome of *B. cenocepacia* H111 (European Nucleotide Archive NCAFQ00000000). In three mutants the transposon was found to be inserted at two different positions within the same gene (Fig. S1) and therefore our analysis identified four independent genes affecting cadmium resistance. The first gene, which was interrupted in mutants 35aA7 and 40G3, was identified as BCAE0587. This gene is predicted to encode a P-type ATPase of the CadA family, members of which have been demonstrated to export lead, cadmium, zinc and mercury and are known as major determinants of HM resistance in many, mostly Gram-positive, bacteria (Nies, 2003). In mutant 3G12 the transposon had inactivated BCAE0365, encoding a putative Δ9 fatty acid desaturase. Fatty acid desaturases catalyse the formation of a double bond in fatty acyl chains and are thus essential for proper membrane function in bacteria (Shanklin and Cahoon, 1998), suggesting that the higher cadmium susceptibility of this transposon mutant was a consequence of altered membrane permeability or stability. In two mutants (35aA12 and 8D3) the transposon had inserted at different positions in BCAE1998, which encodes a hypothetical protein that was identified in *Ralstonia eutropha* H16 as a DNA repair protein involved in the RecF pathway (Morimatsu and Kowalczykowski, 2003). As cadmium has been reported to induce DNA breakage (Mitra and Bernstein, 1978; Badisa *et al*., 2007), it is likely that the higher cadmium sensitivity of the two mutants is due to a defect in DNA repair. In fact, the two mutants were also found to be much more sensitive to UV radiation than the wild type (data not shown). The last gene identified in our screen was BCAE3201 (mutants 7G10 and 8E11), which encodes a protein that is homologous to TolR. The TolR protein is part of the Tol multiprotein complex, which plays a key role in the maintenance of outer membrane integrity and cell morphology in Gram-negative bacteria (Llamas *et al*., 2000). As with mutant 3G12, the cadmium sensitivity of these mutants is likely caused by a destabilized cell envelope.

In conclusion, three of the identified genes required for full cadmium resistance are essential for outer membrane biogenesis and integrity (BCAE0365, BCAE3201) or DNA repair (BCAE1998) and thus do not confer a specific resistance mechanism but rather affect cadmium uptake or the overall fitness of the cell. In this context it is important to note that although growth of these mutants was clearly impaired, it was not entirely abolished. This is in stark contrast to the two *cadA* mutants, which showed no growth at all in LB medium supplemented with 1 mM $CdSO_4$, indicating that this gene is the major player in cadmium resistance of *B. cenocepacia*. We therefore focused our subsequent investigations on *cadA*.

CadA confers resistance to cadmium, zinc and lead, but not to nickel, cobalt and copper

We next constructed a defined *cadA* knock-out mutant, H111Δ*cadA*, as outlined in Appendix S1 and this strain was used along with the transposon insertion mutant to assess the role of *cadA* in HM resistance (Table S1). As a control we complemented mutant 35aA12 with a cosmid carrying the *cadA* wild type allele (Table S2). The various strains were tested for growth in a mineral base medium containing different concentrations of cadmium, zinc, lead, nickel, cobalt and copper. Growth of both *cadA* mutants was severely impaired in the presence of 2 mM cadmium, 2 mM lead and 5 mM zinc when compared with the wild type (Fig. 1). Expectedly, the growth defect was at least partially restored with the complemented mutant. In contrast, inactivation of *cadA* did not affect nickel, copper or cobalt resistance. While increasing cobalt concentrations led to decreased growth in all the strains tested, no growth inhibition was observed when the strains were grown with up to 5 mM nickel or copper (Fig. 1D; data not shown). The fact that mutants tolerated higher cadmium concentrations than in the original screening is likely to be due to the different

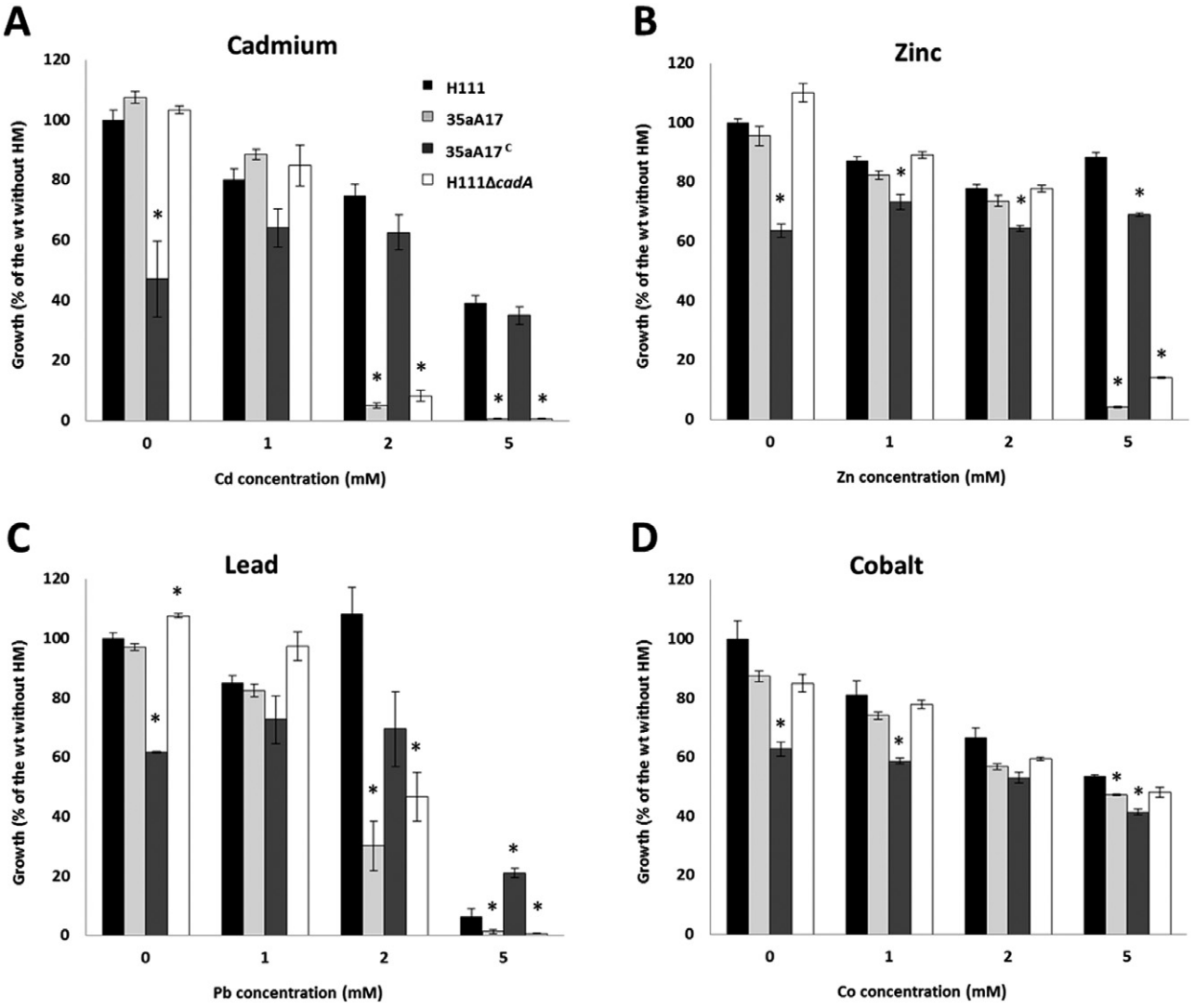

Fig. 1. Growth of the wild type *B. cenocepacia* H111 (black bars), the 35aA12 transposon mutant (35aA17, light grey), the complemented transposon mutant (35aA17^C, dark grey) and a defined *cadA* mutant (H111Δ*cadA*, white) was assessed in mineral base medium (adapted from Van Nostrand *et al*., 2005) containing different HMs in concentrations varying from 1 to 5 mM. Strains were grown overnight in mineral base medium at pH 7. The cultures were adjusted to an OD_{600} of 0.005 in 96-well microtitre plates containing 200 µl of minimal base medium (pH 7) amended with different concentrations of $CdSO_4$ (A), $ZnSO_4$ (B), $Pb(NO_3)_2$ (C) or $CoCl_2$ (D). Plates were incubated overnight at 37°C and growth was measured by assessing the optical density at 600 nm. Results shown are percentages of the OD_{600} obtained for the wild type in the absence of HM. Stars indicate statistical differences with the wild type (Student's *t*-test, $P < 0.001$, $n = 4$).

media used (mineral base medium versus LB) and/or to the biofilm lifestyle, which was favoured by the microtitre plate assay and might lead to a higher level of protection against HMs (Harrison *et al*., 2007).

Induction of cadA *expression is HM-specific and pH-dependent*

To investigate expression of *cadA* we constructed a fusion of the *cadA* promoter region (P_{cadA}) to the GFP. As expected, expression was strongly induced in the presence of 1 mM cadmium, lead and zinc, but not by nickel, copper and cobalt (Fig. 2A). In agreement with previous reports (Van Nostrand *et al*., 2005; Worden *et al*., 2009), we noticed that HM resistance of *B. cenocepacia* H111 was higher when cells were grown at pH 5 than at pH 7 (data not shown), which is likely due to an increased competition of HM cations with protons at the cell surface (Franklin *et al*., 2000). To test whether pH also affected the induction of the P_{cadA}–*gfp* fusion we cultured the sensor strain at pH 5 and pH 7 in the presence of various $CdSO_4$ concentrations. These experiments revealed that at pH 7 the fusion was not only induced earlier but also at much higher levels than at acidic pH

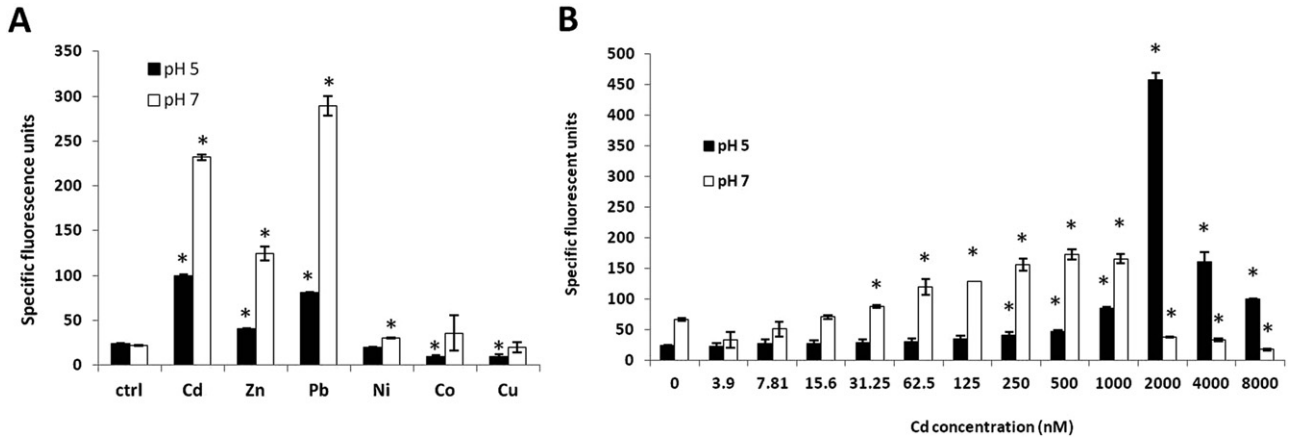

Fig. 2. Induction of *cadA* expression by different HMs. A plasmid carrying a transcriptional fusion of the *cadA* promoter with *gfp* was transferred into *B. cenocepacia* H111. An overnight culture of the strain was harvested, washed and inoculated into mineral base medium supplemented with 20 μg ml^{-1} gentamicin (final OD_{600} of 0.001) and 1 mM of either $CdSO_4$, $ZnSO_4$, $Pb(NO_3)_2$, $NiCl_2$, $CuSO_4$ or $CoSO_4$. Following 18 h incubation at 37°C the OD_{600} was adjusted to 2.0 with 0.9% NaCl solution. Green fluorescence was measured at 528 nm in a spectrofluorometer microplate reader. Specific fluorescence units (fluorescence divided by OD_{600}) are shown. Expression of *cadA* in acidic (black bars) or neutral (white bars) mineral base medium containing 1 mM of either HM (A). $CdSO_4$ concentrations required for induction of *cadA* expression at acidic (black bars) or neutral (white bars) pH (B). Stars indicate statistically different values from the respective HM-free controls (Student's *t*-test, $P < 0.001$, $n = 2–3$).

(Fig. 2B), suggesting a quicker and higher stress response at neutral pH. These results demonstrate that expression of *cadA* is specifically triggered by cadmium, zinc and lead and that the expression level is dependent on pH.

Monitoring single cell expression of the P_{cadA}–gfp fusion on the roots of the HM-hyperaccumulating plant A. halleri

Previous work has demonstrated that various organic and inorganic pollutants can be detected *in situ* by the aid of bacterial biosensors (van der Meer and Belkin, 2010). Although biosensors responding to various HMs with high sensitivity have been developed (Ivask *et al*., 2009), they have not been used for single cell expression studies, which may allow investigations of the spatial distribution of HMs in the sample. To test whether the P_{cadA}–*gfp* fusion is suitable for single cell analysis, we inoculated the reporter strain on the roots of *A. halleri* grown in hydroponic cultures in the absence or presence of 1 mM $CdSO_4$. In order to be able to localize the sensor strain on the root surface, it was chromosomally tagged with the red fluorescent protein (RFP). After 6 days, plant roots were inspected by confocal laser scanning microscopy. The sensor strain was found to form micro-colonies on the roots, independently of the presence or absence of cadmium (Fig. 3). Expectedly, only on the root surface of plants grown in medium supplemented with $CdSO_4$, the sensor cells also showed strong green fluorescence (Fig. 3). These data provide proof of principle that the reporter strain is well suited to *in situ* detect cadmium in a complex environmental sample at the resolution of a single bacterial cell.

Conclusions

Although *Burkholderia* species are intrinsically resistant to HM and have been frequently retrieved from HM-contaminated environments (Kunito *et al*., 1997; Brim *et al*., 1999; Palmroth *et al*., 2007; Jiang *et al*., 2008; Lazzaro *et al*., 2008; Kuffner *et al*., 2010), knowledge on the underlying molecular mechanisms of HM resistance is scarce. Here we have shown that the P1-type ATPase CadA (BCAE0587) of *B. cenocepacia* H111 mediates resistance to cadmium, lead and zinc. CadA is highly conserved within the genus *Burkholderia*, including the plant endosymbiont *Burkholderia phytofirmans*, the plant pathogens *Burkholderia glumae* and *Burkholderia gladioli* and the human pathogen *Burkholderia pseudomallei*. Moreover, the *cadA* orthologues are in most species located close to the origin of replication of the main chromosome, a position that is typical for highly conserved core genes. As expected, the *cadA* gene is present in all sequenced *Burkholderia* genomes. Orthologues of *cadA* have also been described in various other bacteria, where they are often located on mobile elements, indicating that they are part of the accessory genome, i.e. only present in some adapted strains of a species. Although *Burkholderia* sp. is commonly found in soil, particularly associated with plant roots, and thus may frequently encounter high HM concentrations, it remains to

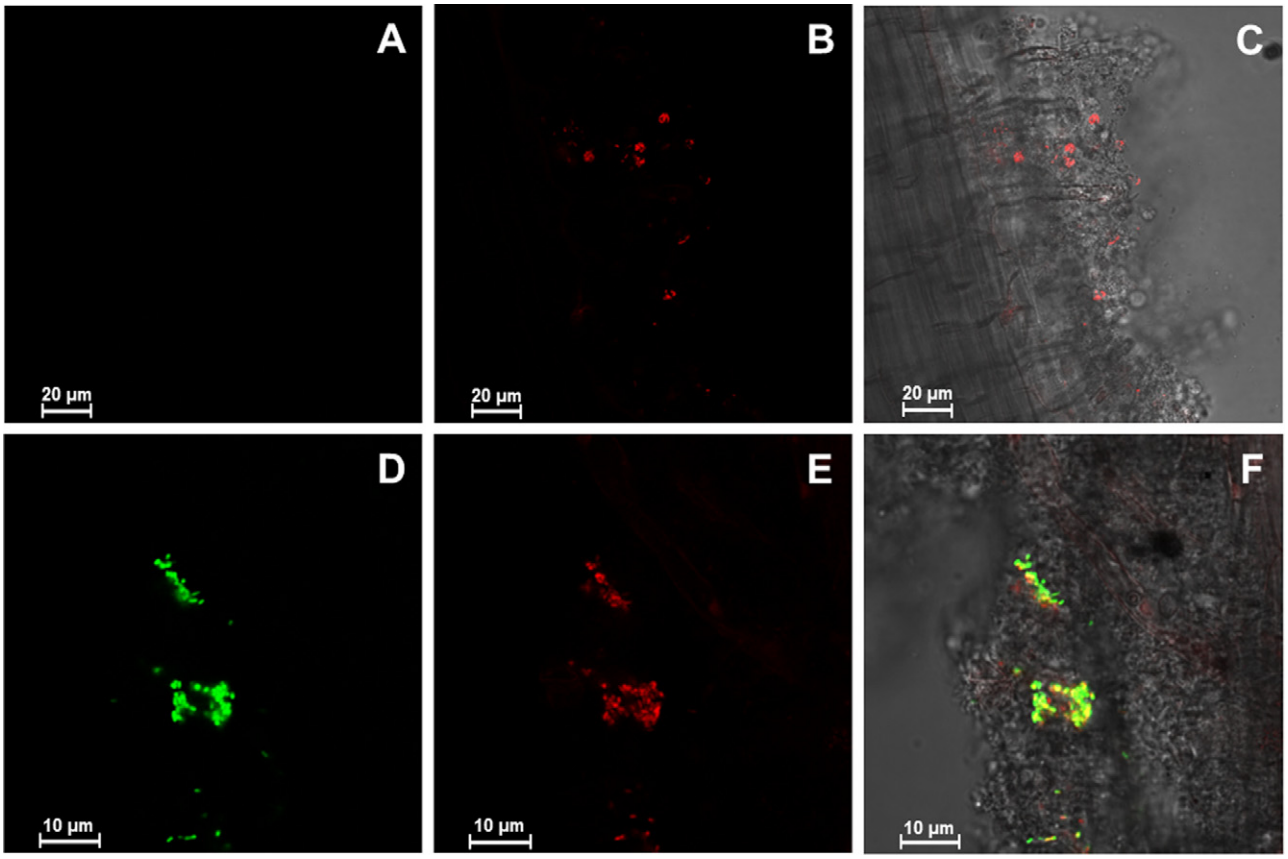

Fig. 3. Monitoring *cadA* expression on the roots of *A. halleri*. Seeds of *A. halleri* were surface sterilized, germinated and grown for 4 weeks on half-strength MS medium, after which they were transferred to hydroponic cultures. After 5 weeks the plants were exposed to 1 mM $CdCl_2$ and inoculated with RFP-tagged cells of *B. cenocepacia* H111 harbouring a plasmid carrying a *cadA::gpf* transcriptional fusion. Following 1 week of incubation the roots were inspected by confocal laser scanning microscopy (DM5500Q, Leica). Representative regions of roots from plants grown in either absence (A, B, C) or presence (D, E, F) of 1 mM $CdCl_2$ are shown. Green fluorescence (A, D) indicates *cadA* promoter activity; red fluorescence (B, E) shows the position of cells on the root surface. C and F show double exposure pictures that were overlaid with a phase contrast image.

be elucidated why CadA-mediated HM resistance is so highly conserved within the genus *Burkholderia*.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Identification of genes involved in cadmium resistance in *B. cenocepacia* H111. Screening of a Tn*5*-insertion mutant library resulted in seven mutants that had lost the ability to grow on LB medium supplemented with 2 mM $CdSO_4$. The DNA sequences flanking the transposon were determined using arbitrary PCR, essentially as described by O'Toole and Kolter (1998). Amplicons were sequenced and reads were mapped to the genome of *B. cenocepacia* H111.

Table S1. Strains used in this study.
Table S2. Plasmids used in this study.
Appendix S1. Experimental procedures.

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