Aconitase B Is Required for Optimal Growth of Xanthomonas campestris pv. vesicatoria in Pepper Plants

Janine Kirchberg1, Daniela Böttner2, Barbara Thiemer1, R. Gary Sawers1*

1 Department of Microbiology, Institute of Biology, Martin-Luther University Halle-Wittenberg, Halle (Saale), Germany, 2 Department of Genetics, Institute of Biology, Martin-Luther University Halle-Wittenberg, Halle (Saale), Germany

Abstract

The aerobic plant pathogenic bacterium Xanthomonas campestris pv. vesicatoria (Xcv) colonizes the intercellular spaces of pepper and tomato. One enzyme that might contribute to the successful proliferation of Xcv in the host is the iron-sulfur protein aconitase, which catalyzes the conversion of citrate to isocitrate in the tricarboxylic acid (TCA) cycle and might also sense reactive oxygen species (ROS) and changes in cellular iron levels. Xcv contains three putative aconitases, two of which, acnA and acnB, are encoded by a single chromosomal locus. The focus of this study is aconitase B (AcnB). acnB is co-transcribed with two genes, XCV1925 and XCV1926, encoding putative nucleic acid-binding proteins. In vitro growth of acnB mutants was like wild type, whereas in planta growth and symptom formation in pepper plants were impaired. While acnA, XCV1925 or XCV1926 mutants showed a wild-type phenotype with respect to bacterial growth and in planta symptom formation, proliferation of the acnB mutant in susceptible pepper plants was significantly impaired. Furthermore, the deletion of acnB led to reduced HR induction in resistant pepper plants and an increased susceptibility to the superoxide-generating compound menadione. As AcnB complemented the growth deficiency of an Escherichia coli aconitase mutant, it is likely to be an active aconitase. We therefore propose that optimal growth and survival of Xcv in pepper plants depends on AcnB, which might be required for the utilization of citrate as carbon source and could also help protect the bacterium against oxidative stress.

Introduction

Pathogenic bacteria of the genus Xanthomonas infect both monoe- and dicotyledonous plants and are responsible worldwide for considerable losses in plant productivity [1,2,3]. Xanthomonas campestris pv. vesicatoria (Xcv) causes bacterial spot disease on pepper and tomato plants and is a model bacterium for the study of bacterial pathogenesis [1]. It enters the plant through openings such as wounds or through stomata and colonizes the intercellular spaces between plant cells. Virulence of the bacterium depends on the type III secretion system (T3SS) [4,5], which injects a number of effector proteins into plant cells. As well as being involved in the development of disease symptoms, many of these effector proteins probably interfere with the host defense mechanisms. To establish effective colonization of the host the bacterium not only has to acquire growth substrates successfully but presumably also has to deal with a number of defense responses initiated by the host in response to infection. Amongst these, iron-restriction and an induced oxidative stress response are likely to be important [6,7]. Comparatively little is known regarding how Xcv grows in planta, particularly with regard to substrate utilization but also with regard to the strategies employed to combat host defense mechanisms. Because Xcv is an obligate aerobe, this significantly increases the spectrum of carbon sources available to the bacterium for biosynthesis of new cell material and energy conservation. These carbon sources include a number of organic acids and amino acids, which can be oxidized by the tricarboxylic acid (TCA) cycle. Current evidence indicates that citrate is one important organic acid that can be used by the bacterium as a carbon source in the plant apoplast [8,9,10]. A key enzyme of the TCA cycle that not only catalyzes the interconversion of citrate and isocitrate, but also has a role in monitoring iron homeostasis and sensing oxidative stress is aconitase (Acn). Members of the Acn protein family are large monomeric, or occasionally dimeric [11], proteins that have a labile [4Fe-4S] cluster, which is required for enzyme activity. Because of the labile nature of the [4Fe-4S] cluster aconitases can function as sensors of both iron limitation and oxidative stress and this has meanwhile been demonstrated for a number of organisms [12]. Upon disassembly of the [4Fe-4S] cluster the apo-protein (termed the iron-responsive protein, IRP) adopts an alternative conformation that allows it to regulate gene expression at a post-transcriptional level. Processes regulated by apo-Acn include the oxidative stress response [13], sporulation in Bacillus subtilis [14] and stationary phase survival in Staphylococcus aureus [15]. A close association between Acn, iron deficiency and bacterial virulence has also been demonstrated for several bacterial pathogens [16,17].
Bacterial AcnA fall into two main classes, AcnA and AcnB [12,16]. Although AcnA and AcnB have related biochemical activities [19], they exhibit only limited amino acid sequence similarity with each other. They have different domain organisation and AcnB proteins have an extra dimerization domain required both for protein-protein interaction and mRNA-binding activity [20]. In many bacteria AcnB is the main aconitase functional in the TCA cycle and it is sensitive to oxidative stress. AcnA on the other hand is induced in the stationary phase in response to iron and oxidative stresses [21,22].

Circumstantial evidence for an important role of aconitase in regulating pathogenicity factor gene expression, e.g. production of extracellular enzymes and polysaccharides, in the plant pathogenic bacterium X. campestris pv. campestris was provided when an analysis of an rpfA mutant (regulation of pathogenicity factors A) proved to have a mutation in the gene encoding AcnA [23]. Comparative genome analysis reveals that both X. campestris pv. campestris and Xcv each encode three Acns [24,25]. An acnA gene, equivalent to rpfA [23], is divergently transcribed from the acnB gene while a second acnA2 gene (XCV1158) is located at a separate location on the genome in a cluster of genes predicted to encode enzymes of methylcitrate metabolism [24]. In this study we examined the potential role of AcnB in the pathogenesis and growth of Xcv in planta. Our studies reveal a requirement for AcnB to allow optimal growth of Xcv in pepper plants but not in liquid culture.

Results

The XCV1925-XCV1926-acnB Genes from Xcv are Co-transcribed and Conserved in the Genus Xanthomonas

The genes encoding AcnA and AcnB are divergently transcribed in the genus Xanthomonas (Fig. 1). Immediately upstream of acnB are two genes, termed XCV1925 and XCV1926. While XCV1926 is conserved in all species of the genus Xanthomonas, XCV1925 is absent in Xanthomonas fuscans. A similar gene organisation is also observed in the plant pathogenic bacterium Xylella fastidiosa and in Stenotrophomonas maltophilia, both of which belong to the family Xanthomonadaceae. This gene order is not conserved in other gammaproteobacteria such as Escherichia coli (Fig. 1).

The XCV1925 gene encodes a predicted protein of 8.7 kDa that belongs to the AbdB family of transition-state regulators [26], while XCV1926 encodes a predicted member of the VapC/PIN family of ribonucleases [27,28]. AbdB proteins respond to a variety of environmental stimuli and regulate processes such as spore development, competence, and biofilm formation [29,30,31]. XCV1925 and XCV1926 overlap by 4 bp, while XCV1926 and acnB are separated by a 50-bp intergenic region.

To determine whether the XCV1925-XCV1926-acnB genes are co-transcribed and form an operon we performed RT-PCR (reverse transcriptase-polymerase chain reaction) with total RNA isolated from Xcv strain 85-10 grown aerobically in shake-flask culture to the mid-exponential phase of growth in complex NYG (nutrient yeast glycerol) medium. A 790-bp cDNA fragment spanning acnB, XCV1925 and XCV1926 was amplified, suggesting that all three genes are co-transcribed (Fig. 2A). This proposal is furthermore supported by the findings of a recent global transcriptome analysis of Xcv [32] in which the authors identified a single cDNA species encompassing the XCV1925, XCV1926 and acnB genes. Taken together, these findings indicate that a putative functional relationship exists between XCV1925, XCV1926 and AcnB and therefore we decided to direct the main focus of our study on the AcnB enzyme.

Expression of AcnA and AcnB Occurs in Both Exponential and Stationary Phase Cultures

In order to analyze when the Acns of Xcv are expressed during in vitro growth, a semi-quantitative RT-PCR analysis of the acnB transcript, and as a control the rpfA transcript, was performed. Total RNA was isolated from exponential and stationary phase cultures of Xcv growing in rich medium and aliquots were analyzed by RT-PCR (Fig. 3A). The results show that transcripts from both acnB and the divergently transcribed acnA genes were detectable in both stages of growth. Slightly reduced levels of the acnA transcripts were detected in stationary phase cultures compared with exponentially growing cells, while the opposite was the case for acnB transcript levels. As a further control we analyzed the transcript levels of a second acnA gene (XCV1158), termed acnA2, which is located elsewhere on the chromosome of Xcv, and which encodes a predicted methylcitrate dehydratase that showed similar levels of transcript in both exponential and stationary phase cells and thus acted as a loading control (Fig. 3A).

In addition to transcript studies, we analyzed the amounts of AcnB protein by immunoblotting in Xcv cells from exponential or stationary phase cultures. As the putative AcnB protein from Xcv shares 72% overall amino acid identity and 84% similarity with the deduced amino acid sequence of AcnB from E. coli, we wondered whether both proteins might share immunogenic epitopes. Indeed, we could show that anti-AcnB antibodies from E. coli cross-reacted with an approximately 92-kDa polypeptide in extracts of Xcv (Fig. 3B), which is in close agreement with deduced molecular weight of AcnB from Xcv of 92,659. The amount of AcnB in extracts derived from stationary phase cultures was similar to that in extracts from exponential phase cells. A second cross-reacting polypeptide that migrated with an approximate molecular mass of 86-kDa was also detected. This polypeptide possibly represents a degradation product of full-length AcnB (Fig. 3B).

In order to demonstrate that the cross-reacting polypeptide was indeed AcnB from Xcv we constructed two distinct acnB deletion mutants of Xcv (Fig. 2A), Strain 85-10ΔacnB has a deletion encompassing codon 1 to the termination codon of the acnB gene, while strain 85-10ΔXCV1925-26acnB carries a deletion in the complete XCV1925, XCV1926 and acnB genes (see Methods; Table 1). The strong cross-reacting 92-kDa polypeptide was absent in extracts derived from strains 85-10ΔacnB and 85-10ΔXCV1925-26acnB, thus demonstrating that this polypeptide indeed represented AcnB from Xcv (Fig. 3B). The weak, cross-reacting polypeptide observed in extracts from stationary phase cultures was similar to that in extracts from exponential phase cells. A second cross-reacting polypeptide that migrated with an approximate molecular mass of 86-kDa was also detected. This polypeptide possibly represents a degradation product of full-length AcnB (Fig. 3B).

Aconitase B is not Required for in Vitro Growth of Xcv when Sucrose is the Carbon Source

Two further mutant derivatives of Xcv strain 85-10 were constructed in which the acnA gene or the two small genes XCV1925 and XCV1926 were deleted (see Fig. 2 and
The resulting mutant strains 85-10 ΔacnA and 85-10 ΔXCV1925-26, along with strains 85-10 (wild-type), 85-10 ΔacnB and 85-10 ΔXCV1925-26 ΔacnB, all grew with similar rates and attained similar final optical densities in vitro in NYG medium (data not shown). Growth studies performed in minimal medium with sucrose showed that the strains 85-10, 85-10 ΔacnB and 85-10 ΔXCV1925-26 ΔacnB also showed similar growth phenotypes (Fig. 4A). Moreover, strain 85-10 ΔacnA also grew like the wild-type under these conditions (data not shown).

The lack of an in vitro growth phenotype for the 85-10 ΔacnA mutant is in agreement with previous observations for an rpfA (acnA) mutant of X. campestris pv. campestris [23].

Figure 2. Co-transcription of xcv1925, xcv1926 and acnB. A. Schematic representation of the deletions introduced in the genes at the acnB locus. 1. represents the extent of the deletion in strain 85-10ΔacnB; 2. represents the deletion in 85-10ΔXCV1925-26 ΔacnB; 3. represents the deletion in 85-10ΔacnA and 85-10ΔXCV1925-26 ΔacnB. B. RT-PCR analysis of the XCV1925-XCV1926-acnB transcript. Total RNA was isolated and analyzed as described in the Methods using oligonucleotide primers r-secacnB and f-sec3565 (Table S1). Lane 1, DNA size standards; lane 2, PCR product with cDNA as template; lane 3, including cDNA (lane 2) and minus reverse transcriptase (lane 3). The lane labelled C represents the products of a PCR with genomic DNA as template and the primer pairs described above. The lane on the left of each gel segment shows a DNA size standards. Analysis of 16S rRNA revealed equivalent loading (data not shown). B. A Western blot is shown in which 25 μg of protein derived from crude extracts of the strains indicated were separated in 8% SDS-PAGE and transferred to nitrocellulose membranes and subsequently probed with antibodies raised against E. coli AcnB. The location of AcnB is indicated and Exp. and Stat. represent samples from exponential and stationary phase cultures, respectively. The location of molecular mass markers in kDa is indicated on the right of the Figure. doi:10.1371/journal.pone.0034941.g002
Xcv Strain 85-10ΔacnB Shows Restricted Growth in Minimal Medium with Citrate

As citrate is the substrate for aconitase and the bacterium encodes a citrate transporter, we next compared growth of strains 85-10 and 85-10ΔacnB in vitro in minimal medium with 15 mM citrate as sole carbon source (Fig. 4B). The wild-type strain 85-10 grew more poorly than with sucrose as a carbon source (compare Fig. 4A and Fig. 4B) but it nevertheless attained a final optical density at 600 nm of approximately 0.35. Without addition of a carbon source no growth of the wild-type was observed (data not shown). Strain 85-10ΔacnB showed a clearly reduced ability to grow with citrate compared with the wild-type strain (Fig. 4B). Taken together, the findings of the in vitro growth studies indicate that, although the acnB mutation did not affect growth with sucrose as a carbon source growth in the presence of citrate was affected.

Strains Lacking AcnB Show Delayed Growth and Symptom Formation in Pepper Plants

Citrate is abundant in the tomato apoplast [8] and recent studies have shown that expression of the citB gene of Xcv, encoding a citrate transporter, is up-regulated in planta [9,10]. Therefore, we investigated the consequences of the different gene deletions on growth of the respective bacterial strain in planta. Strains 85-10, 85-10ΔacnA, 85-10ΔacnB, 85-10ΔXCV1925-26 and 85-10ΔXCV1925-26ΔacnB were inoculated into leaves of the susceptible pepper line Early California Wonder (ECW). Strain 85-10ΔacnA and the wild type 85-10 showed similar growth in susceptible plants (Fig. 5A). As a negative control we analyzed growth of strain 85-10ΔhrcN [33], which lacks the ATPase HrcN of the T3S system and was therefore strongly impaired in in planta proliferation. In contrast to the growth phenotypes of strains 85-10 and 85-10ΔacnA, strain 85-10ΔacnB displayed a clearly reduced ability to grow in the plant apoplast (Fig. 5A). The growth of the acnB mutant was, however, not as strongly reduced compared with growth of the hrcN mutant. In a similar experiment, growth of strains 85-10ΔXCV1925-26 and 85-10ΔXCV1925-26ΔacnB were compared with growth of strain 85-10 (Fig. 5B). Strain 85-10ΔXCV1925-26ΔacnB showed a similarly reduced growth phenotype to that observed for strain 85-10ΔacnB. In contrast, however, strain 85-10ΔXCV1925-26 grew like the wild-type strain 85-10 (Fig. 5B). This result indicated that the reduced growth phenotype of strain 85-10ΔXCV1925-26ΔacnB in planta was caused solely by the acnB mutation and that the additional deletion of the XCV1925-XCV1926 genes had no effect on growth.

The reduced growth of strain 85-10ΔacnB in planta could be complemented by introduction of plasmid pL6acnB, which encodes AcnB into the mutant (Fig. 5C). The slightly impaired growth in planta we observed for strains containing the plasmid pLAF6 accounted for the similarly poor growth phenotype of 85-10ΔacnB/pLAF6 and 85-10ΔhrcN. Taken together these results indicate that AcnB is required for optimal growth of Xcv in susceptible pepper plants.

Strains Lacking acnB Show Delayed Appearance of Disease Symptoms

Strains 85-10ΔacnB and 85-10ΔXCV1925-26ΔacnB were next analyzed to determine whether they were affected in the induction of disease symptoms in the ECW pepper line or in the ability to induce
Strains Lacking Aconitase B are more Sensitive Towards the Superoxide-generating Compound Menadione

Aconitase B enzymes have a [4Fe-4S] cluster that is sensitive to oxidative stress [12,13,43]. Exposure of strains 85-10, 85-10ΔacnB and 85-10ΔXCV1925-26acnB to the superoxide-generating chemical menadione revealed that both acnB mutants were more sensitive to 50 μM menadione than the wild-type strain 85-10 (Fig. 8). Increasing the concentration of menadione to 100 μM made this difference much more apparent and while for the wild-type strain 85-10 after 4 days' incubation 90% of the bacteria survived, survival of both mutants was severely impaired and attained levels of between 10 and 20% (Fig. 8). This result suggests that AcnB might have a role in either sensing changes in superoxide levels or protecting Xcv from the deleterious effects of oxidative stress. Notably, E. coli acnB mutants also show increased sensitivity toward superoxide-generating chemicals such as methyl viologen [44]. In contrast to the phenotype of an E. coli acnB mutant, however, strain 85-10ΔacnB failed to reveal a difference in survival when compared with strain 85-10 after exposure to 5 mM hydrogen peroxide (data not shown). These findings demonstrate that although certain phenotypes of Xcv acnB mutants are common to those reported for other bacteria, nevertheless, clearly different phenotypes are evident for the Xcv acnB mutants.

AcnB from X. Campestris pv. Vescitaria is functional in E. coli

In many bacteria aconitases are differentially regulated in response to the prevailing growth conditions. For example, in E. coli AconB is the main TGA cycle enzyme and it is functional in the exponential phase of growth, while AconA is switched on during oxidative stress and in the stationary phase [21,44]. Because of the strong dependence on AconB during the exponential growth phase E. coli acnB mutants show a reduced growth phenotype in liquid culture, while acnA mutants show no growth phenotype [45]. This phenotype provided the opportunity to demonstrate whether AconB from E. coli and Xcv are functionally interchangeable. For this, the acnB gene from Xcv was cloned and under control of the tac promoter in-frame with a C-terminal c-Myc epitope-encoding sequence into the expression vector pBRM [46] (see Methods).
The resulting plasmid pBRM\textit{acnB}, when introduced into an \textit{E. coli} \textit{acnB} mutant, restored the aerobic growth rate of the mutant in liquid culture to a rate similar to that of the wild type (Fig. 9). This result indicates that the two AcnB enzymes are functionally interchangeable and that the C-terminal c-Myc-tag on AcnBXcv did not impair enzyme function. The lack of an observable \textit{in vitro} growth phenotype for strain 85-10\textit{DacnB}, however, contrasts sharply the growth phenotype of an \textit{E. coli acnB} mutant [45] (see also Fig. 3).

**Discussion**

The findings of this study demonstrate that the AcnB enzyme of \textit{Xcv} is required for optimal growth of the bacterium in the apoplast of pepper plants. This is of significance because \textit{acnB} mutants of...
Xcv show no growth phenotype when they are grown in vitro in shake cultures in the presence of sugar substrates. Reduced growth in pepper plants suggests that the bacterium utilizes citrate as one of its carbon sources in planta. Although not analyzed so far for Xcv, apoplastic metabolite studies performed with tomato have identified citrate, along with succinate, as the main organic acid present in apoplastic fluid [8]. Both of these substrates require the TCA cycle to enter primary metabolism. Moreover, the importance of citrate as an apoplastic substrate of Xcv is exemplified by the findings of two studies. In the first, expression of the citH gene, encoding a citrate transporter in Xcv, was shown to be up-regulated specifically in tomato [9]. In a more recent second study, it was demonstrated to be required for citrate uptake by the bacterium when it is growing in tomato [10]. Taken together, these observations are consistent with citrate, the substrate of the AcnB enzyme, being a carbon source for Xcv in apoplastic fluid. Our demonstration in this study that in vitro growth of the acnB mutant with citrate was less efficient than the wild-type supports these findings. The availability of TCA cycle intermediates as carbon sources in planta would also explain why plant-pathogenic Xanthomonas species are obligate aerobes, because anaerobic bacteria cannot metabolize these compounds effectively.

The delayed appearance of disease symptoms in the Xcv acnB mutant is a subtle phenotype that is possibly related to the reduced growth of the mutant in the plant apoplast. However, a reduced growth rate does not explain the reduction in the HR phenotype of the acnB mutant. This may be linked to more general effects on the bacterium’s physiology caused by the impaired citric acid cycle of the acnB mutant. The Xcv acnA mutant failed to exhibit either a growth or virulence phenotype in planta, which highlights a potential difference between Xcv and the Xcc pathovar [23].

A further potentially important function for aconitases in pathogenic bacteria is as sensors of oxidative stress, conditions which are often prevalent during host-microbe interactions [6,47,48]. Sensitivity of these enzymes towards oxidative stress is mediated through the iron-sulphur cluster in aconitase, which has an essential catalytic function in allowing citrate conversion to isocitrate. Our observation here that acnB mutants of Xcv are more susceptible toward superoxide-generating chemicals would be consistent with a role for this enzyme in sensing the presence of ROS in planta and possibly in maintaining iron homeostasis [6].

Finally, although we failed to demonstrate under the conditions tested in this study that the aconitase A of Xcv is essential for optimal growth in planta, as has been suggested in a previous study with X. campestris pv. campestris [23], our findings nevertheless show that in the absence of AcnB a further aconitase is likely to be functional in vitro because an acnB mutant retained the ability to grow slowly with citrate while the wild-type failed to grow without carbon source supplementation. Whether this is AcnA or AcnA2 will require the analysis of double knock-out mutants that lack AcnB and either one of the predicted AcnAs.

In summary, our findings underline the general importance of aconitases in plant-microbe interaction. The importance of aconitase B to Xcv might reflect multiple functions of the protein in planta: 1. Aconitase B is a key enzyme for the metabolism of TCA intermediates; 2. AconB possibly has a role in sensing oxidative stress; 3. Although not tested in this study, it is conceivable that aconitase B is involved in monitoring iron homeostasis during growth of the bacterium in the plant. Future studies will focus on elucidating whether AconB indeed performs all of these functions when the bacterium is growing in pepper plants.

Methods

Bacterial Strains, Growth Conditions, and Plasmids

The bacterial strains and plasmids used in this study are described in Table 1. X. campestris pv. vesicatoria strains were cultivated at 30°C in complex nutrient-yeast-glycerol (NYG) medium [49] or in minimal medium A [50] supplemented with sucrose (10 mM) and casamino acids (0.3% w/v). E. coli cells were cultivated at 37°C in Luria-Bertani medium. Plasmids were introduced into E. coli by electroporation or using heat-shock treatment and then into Xcv by conjugation using pRK2013 as a helper plasmid in triparental matchings [51]. Antibiotics were added to culture media at the following final concentrations:

Figure 8. X. campestris pv. vesicatoria strain 85-10::acnB has increased sensitivity to the superoxide-generating agent menadione. Dilutions of 10⁻³ of exponential phase cultures (OD₆₀₀ = 0.6) of the Xanthomonas strains indicated were spotted on NYG agar plates containing 50 µM and 100 µM menadione. Bacterial colonies surviving the treatment were counted after 24 and 48 h of incubation at 30°C and CFU were expressed as surviving fraction in percent. Strain 85-10 (black columns), strain 85-10::acnB (white columns) and strain 85-10::XCV1925-26acnB (gray columns).

doi:10.1371/journal.pone.0034941.g008

Figure 9. Aconitase B from Xcv functionally complements an E. coli acnB mutation. The E. coli wild type W3110 (filled squares), the acnB mutant JRG3258 (filled circles) and JRG3258 transformed with plasmid pBRMacmanB (filled triangles) were grown aerobically in LB broth as described in the Methods section. The standard error is shown for each experiment.

doi:10.1371/journal.pone.0034941.g009
ampicillin 100 μg/ml, kanamycin 25 μg/ml, rifampicin 100μg/ml, spectinomycin 100μg/ml, tetracycline 5μg/ml, cyclohexamide 50μg/ml.

**Plant Material and Plant Inoculations**

The near-isogenic pepper cultivars Early Cal Wonder (ECW) and ECW-10R [52], which contains the BtI resistance gene [35], were grown and inoculated with Xcv as described [53]. Bacteria were inoculated into the intercellular spaces of fully expanded leaves of 5–6 week-old plants using a needleless syringe at a concentration of 10^7 CFU ml^{-1} (OD = 0.01) to 4 leaves of 5–6 week-old plants using a needleless syringe at were inoculated into the intercellular spaces of fully expanded leaves of 5–6 week-old plants using a needleless syringe at 5 days after inoculation and the HR over a period of 2 days.

For in planta growth curves, bacteria were inoculated at a density of 10^4 CFU/ml into leaves of susceptible pepper ECW plants (age of 5–6 weeks). Bacterial growth was examined as described [53]. Experiments were repeated at least three times and each time with three separate plants.

**Construction of Deletion Mutants**

To delete genes, approximately 1.0 kb of DNA sequences flanking the gene of interest were amplified by PCR using the primers listed in Table S1 and with Xcv genomic DNA as template. The PCR products to delete acnB were digested with XbaI/ HindIII and HindIII/ApaI and the products to delete XCV1925-26-acnB with XbaI/HindIII and HindIII/Sall, followed by ligation into the Xbal/Apal and XbaI/Sall sites, respectively, of the suicide plasmid pOK1. Similarly, the Xbal/HindIII- and HindIII/BamHI-digested PCR products to delete XCV1925-26XCV1926 and the Xbal/HindIII- and HindIII/ApaI-digested products used to delete acnA were ligated into XbaI/BamHI and XbaI/Apal sites of the pOK1 vector, respectively. The resulting constructs pOKΔacnB, pOKΔ1925-26acnB, pOKΔ1925-26 and pOKΔacnA were conjugated into strain 85-10 as described [54]. Double-crossover events led to generation of strains 85-10ΔacnB, 85-10ΔXCV1925-26ΔacnB, 85-10ΔXCV1925-26 and 85-10ΔacnA. The extent of the acnB gene deletion 85-10ΔacnB included from 1 bp prior to the translation initiation codon to 3 bp after the terminations codon; the deletion in 85-10ΔXCV1925-26 started 6 bp after the translation initiation codon of XCV1925 and ended 10 bp after the terminations codon of XCV1926; the deletion in strain 85-10ΔXCV1925-26ΔacnB started 6 bp before the translation initiation codon of XCV1925 and ended 3 bp after the terminations codon of acnB; and the deletion in 85-10ΔacnA started 2 bp before the translation initiation codon of acnA and ended 1 bp after the termination codon.

**RT-PCR Analysis**

Isolation of total RNA for reverse transcriptase PCR (RT-PCR) analysis after growth of the bacteria in NYG medium. RNA extraction and cDNA synthesis were performed as described previously [38]. The transcripts were amplifed by PCR using the gene-specific primers listed in Table S1. Experiments were performed minimally two time and each time with freshly isolated RNA samples.

**Construction of Plasmids**

To enable complementation studies, the acnB and XCV1925-XCV1926-acnB genes were amplified by PCR using the primers listed in Table S1 and genomic DNA from Xcv as template. The PCR products were digested with XbaI/BamHI (in the case of acnB) and HindIII/XbaI (in the case of XCV1925-XCV1926-acnB) and ligated into the XbaI/BamHI and HindIII/XbaI sites, respectively, of pLAFR6 vector. The resulting constructs were transformed in strain E. coli XL1 blue. These transformed E. coli strains were used as donors to conjugate plasmid into strain 85-10ΔacnB and 85-10ΔXCV1925-26-acnB. The acnB gene was also cloned into the Gloden-Gate-compatible vector pBRM by amplification with primers t-pBRM-acnB and r-pBRM-acnB listed in Table S1 and incorporation of appropriate Bsal restriction sites to introduce a C-terminal c-Myc epitope onto AcnB [46].

**Immunoblot Analyses**

For detection of AcnA and AcnB in crude extracts of Xcv, bacteria were cultivated in NYG or minimal media as described above. Samples of cells were harvested from mid-exponential or stationary phase cultures. After cell harvest, cells were resuspended in 2–3 ml of MOPS buffer pH 7.0 and lysed on ice by sonication (30W power for 5 minutes with 0.5 sec pulses). Unbroken cells and cell debris were removed by centrifugation for 15 min at 10 000 × g at 4°C and the supernatant was used as the crude cell extract. Protein concentration was determined as described [55] and aliquots of 50 μg of protein from the crude extract were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% (w/v) polyacrylamide [56] and transferred to nitrocellulose membranes as described [57]. Aconitase was identified using polyclonal antiserum raised against AcnB from E.coli (a kind gift from J. Green, Sheffield, UK) or monoclonal anti-c-Myc antibodies (Roche Applied Science, Mannheim, Germany). Anti-AcnB antiserum was used at a dilution of 1:10,000. Horseradish-peroxidase-labeled conjugate (Goat Anti-Rabbit IgG (H+L)-HRP-conjugate, 1:5000; Biorad, Munich, Germany) was used as secondary antibody-conjugate and the reaction was visualized by enhanced chemiluminescence (Roche Diagnostics, Mannheim, Germany). Immunoblotss were performed at least twice, each time with freshly prepared samples.

**Plate-sensitivity Assay**

The resistance level of the cells to menadione was determined using a modification of the plate-sensitivity assay described previously [47]. Dilutions of 10^{-6} and 10^{-7} of exponential phase cultures (OD_{600} = 0.6) of the Xanthomonas strains to be tested were spotted on NYG agar plates containing 30 μM or 100 μM menadione (Sigma, Munich). Bacterial colonies surviving the treatment were counted after 24 h and 48 h of incubation at 30°C and data are expressed as surviving fraction in percent. The percentage survival was calculated by dividing the number of CFU from plates with menadione by the number of CFU from control plates without menadione. This experiment was performed in triplicate and repeated at 3 times.
Supporting Information

Table S1 Oligonucleotide primers used in this study.

Author Contributions

Conceived and designed the experiments: JK DB BT RGS. Performed the experiments: JK. Analyzed the data: JK DB BT RGS. Wrote the paper: DB RGS.

References

1. Buttmann D, Bonas U (2010) Regulation and secretion of Xanthomonas virulence factors. FEMS Microbiol Rev 34: 167–183.

2. Leyns F, De Cleene M, Swings J, De Ley J (1984) The host range of the genus Xanthomonas. Bot Rev 50: 305–355.

3. Ryan RP, Vorholt FJ, Potnis N, Jones JB, Van Sluys MA, et al. (2011) Pathogenomics of Xanthomonas: understanding bacterium-plant interactions. Nat Rev Microbiol 9: 444–55.

4. Buttmann D, Bonas U (2002) Getting across-bacterial type III effector proteins on their way to the plant cell. EMBO J 21: 5311–5322.

5. Ghosh P (2004) Process of protein transport by the type III secretion system. Microbiol Mol Biol Rev 68: 771–781.

6. Jitjavittipoka T, Sasanaphatheerat S, Vattanaviboon P, Mongkolbun S (2010) Mutations of ferric uptake regulator (fur) impair iron homeostasis, growth, oxidative stress survival, and virulence of Xanthomonas campestris pv. campestris. Arch Microbiol 192: 331–339.

7. Cornelis P, Wei Q, Andrews SC, Vincx T (2011) Iron homeostasis and management of oxidative stress response in bacteria. Metalomics 3: 540–49.

8. Rico A, Preston GM (2008) Pseudomonas syringae pv. tomato DC3000 uses constitutive and auxin-induced nutrient assimilation pathways to catalyze nutrients that are abundant in the tomato apoplast. Mol Plant Microbe Interact 21: 269–282.

9. Tamir-Ariel D, Navon N, Burdman S (2007) Identification of genes in Xanthomonas campestris pv. vesicatoria induced during its interaction with tomato. J Bacteriol 189: 6359–6371.

10. Tamir-Ariel D, Rosenberg T, Burdman S (2011) The Xanthomonas campestris pv. vesicatoria acnB gene is expressed early in the infection process of tomato and is positively regulated by the TcDRE two-component regulatory system. Mol Plant Microbe Interact 24: 57–71.

11. Tsuchiya D, Shimizu N, Tomita M (2009) Cooperativity of two active sites in aconitase B. We are indebted to Ulla Bonas and Max Dow for discussions. We thank Jeff Green for supplying antibodies to E. coli aconitase B. We are indebted to Ulla Bonas and Max Dow for discussions.

12. Rouault TA (2006) The role of iron-regulatory proteins in mammalian iron homeostasis and disease. Nat Rev Endocrinol 2: 439–483.

13. Varghese S, Tang Y, Imlay JA (2003) Contrasting sensitivities of Xanthomonas campestris aconitases A and B to oxidation and iron depletion. J Bacteriol 185: 221–230.

14. Minsavage GV, Dahlbeck D, Whalen MC, Kearny B, Bonas U, et al. (1990) Gene-for-gene relationships specifying disease resistance in Xanthomonas campestris pv. vesicatoria-pepper interactions. Mol Plant Microbe Interact 3: 41–47.

15. Ronald PC, Saakwazie B (1983) The avirulence gene avrBs1 from Xanthomonas campestris pv. vesicatoria encodes a 50-kD protein. Mol Plant Microbe Interact 1: 379–84.

16. Banerjee S, Nandyala AK, Raviprasad P, Ahmed N, Hasnain SE (2007) Iron-dependent RNA-binding activity of Mycobacterium tuberculosis aconitase. J Bacteriol 189: 4046–4052.

17. Koebnik R, Krüger A, Thieme F, Urban A, Bonas U (2006) Specific binding of DB RGS. Conceived and designed the experiments: JK DB BT RGS. Performed the experiments: JK. Analyzed the data: JK DB BT RGS. Wrote the paper: DB RGS.

Acknowledgments

We thank Jeff Green for supplying E. coli strains and antibodies to E. coli aconitase B. We are indebted to Ulla Bonas and Max Dow for discussions. We thank Jeff Green for supplying antibodies to E. coli aconitase B. We are indebted to Ulla Bonas and Max Dow for discussions.
48. Xu XQ, Pan SQ (2000) An Agrobacterium catalase is a virulence factor involved in tumorigenesis. Mol Microbiol 35: 407–414.

49. Daniels MJ, Barber CE, Turner PC, Sawczyc MK, Byrde RJW, et al. (1984) Cloning of genes involved in pathogenicity of Xanthomonas campestris pv. campestris using the broad host range cosmid pLAFR1. EMBO J 3: 3323–3328.

50. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (1996) Current protocols in molecular biology. John Wiley and Sons, Inc., NY.

51. Figurski D, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76: 1648–1652.

52. Kousik CS, Ritchie DF (1998) Response of bell pepper cultivars to bacterial spot pathogen races that individually overcome major resistance genes. Plant Dis 82: 181–186.

53. Bonas U, Schulte R, Fenselau S, Minsavage GV, Staskawicz BJ, et al. (1991) Isolation of a gene-cluster from Xanthomonas campestris pv. vesicatoria that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol Plant Microbe Inter 4: 81–88.

54. Huguet E, Hahn K, Wengelnik K, Bonas U (1998) ΔiapB mutants of Xanthomonas campestris pv. vesicatoria are affected in pathogenicity but retain the ability to induce host-specific hypersensitive reaction. Mol Microbiol 29: 1379–1390.

55. Lowry O, Rosebrough N, Farr A, Randall R (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

56. Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

57. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350–4354.

58. Canteros BI (1990) Diversity of plasmids and plasmid-encoded phenotypic traits in Xanthomonas campestris pv. vesicatoria. Ph.D. thesis. University of Florida, Gainesville, FL, USA.

59. Me´nard R, Sansonetti PJ, Parrot G (1993) Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. J Bacteriol 175: 5989–5906.

60. Bonas U, Stall RE, Staskawicz B (1989) Genetic and structural characterization of the avirulence gene avrBs3 from Xanthomonas campestris pv. vesicatoria. Mol Gen Genet 218: 127–136.

61. Potnis N, Krasileva K, Chow V, Almeida NF, Patil PB, et al. (2011) Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper. BMC Genomics 12: 146.