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RESEARCH PAPER

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CrcZ and CrcX regulate carbon source utilization in *Pseudomonas syringae* pathovar *tomato* strain DC3000

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Small non-coding RNAs (ncRNAs) are important components of many regulatory pathways in bacteria and play key roles in regulating factors important for virulence. Carbon catabolite repression control is modulated by small RNAs (*crcZ* or *crcZ* and *crcY*) in *Pseudomonas aeruginosa* and *Pseudomonas putida*. In this study, we demonstrate that expression of *crcZ* and *crcX* (formerly designated *psr1* and *psr2*, respectively) is dependent upon RpoN together with the two-component system CbrAB, and is influenced by the carbon source present in the medium in the model plant pathogen *Pseudomonas syringae* pv *tomato* DC3000. The distribution of the members of the Crc ncRNA family was also determined by screening available genomic sequences of the Pseudomonads. Interestingly, variable numbers of the Crc family members exist in Pseudomonas genomes. The ncRNAs are comprised of three main subfamilies, named CrcZ, CrcX and CrcY. Most importantly, the CrcX subfamily appears to be unique to all *P. syringae* strains sequenced to date.

Introduction

Molecular and computational analyses have revealed that bacteria contain large numbers of small, non-coding RNA (ncRNA) molecules. Although their function in many cases is unknown, it has become widely accepted that they play critical roles in a variety of cellular processes and regulatory networks by facilitating adaptation to diverse environmental stresses and influencing the production of virulence factors.[1] The majority of ncRNAs are encoded in trans and interact with their RNA targets through an antisense mechanism. In this process, complementary base-pairing with a target mRNA either activates or represses translation of the transcript, or targets the mRNA for degradation and most often requires the RNA chaperone Hfq.[2] Alternatively, some ncRNAs interact directly with a protein target, sequestering the protein and preventing it from performing functions such as activating or repressing translation.[2]

Recently, we identified and characterized two ncRNAs in the genome of *Pseudomonas syringae* pv *tomato* str. DC3000.[3] One ncRNA, designated *psr1* (PSPTO_5668), is located between PSPTO_0964 and PSPTO_0963. The other, *psr2* (PSPTO_5669), is located between PSPTO_1621 and PSPTO_1622. These areas previously had been reported to contain a conserved RNA motif termed gamma-150.[4] Both ncRNAs are significantly larger than the described motif. A third putative member of this family (*psr3*), located between PSPTO_2739 and PSPTO_2740, was also identified. However, *psr3* is disrupted by an insertion element and, therefore, is not expressed in *P. syringae* DC3000.[3] Interestingly, this RNA appears to be fully intact in *P. syringae* B728a and 1448A.

Homologs to DC3000 *psr1* and *psr3* are found in other pseudomonads. In *P. aeruginosa*, a *psr1* homolog (*crcZ*) contains the gamma-150 motif[4] and maps between *pcnB* and genes encoding the CbrA/CbrB two-component sensor-regulator system.[5] The CrcZ ncRNA contains five Crc-binding sites and is thought to sequester the RNA-binding protein Crc under conditions that generate low or no catabolite repression, thus modulating Crc availability and the strength of catabolite repression control (CRC). CrcZ levels also vary according to the carbon source being used and are low in succinate medium (a preferred carbon source for *P. aeruginosa*), at an intermediate level in glucose medium and at a high level in mannitol medium (a non-preferred carbon source and a growth condition that does not generate catabolite repression).[5] *P. putida* contains *crcZ* and an additional ncRNA, *crcY*,[6] homologous to *psr3* in DC3000. The *crcZ* and *crcY* ncRNAs were found to function similarly and modulate levels of

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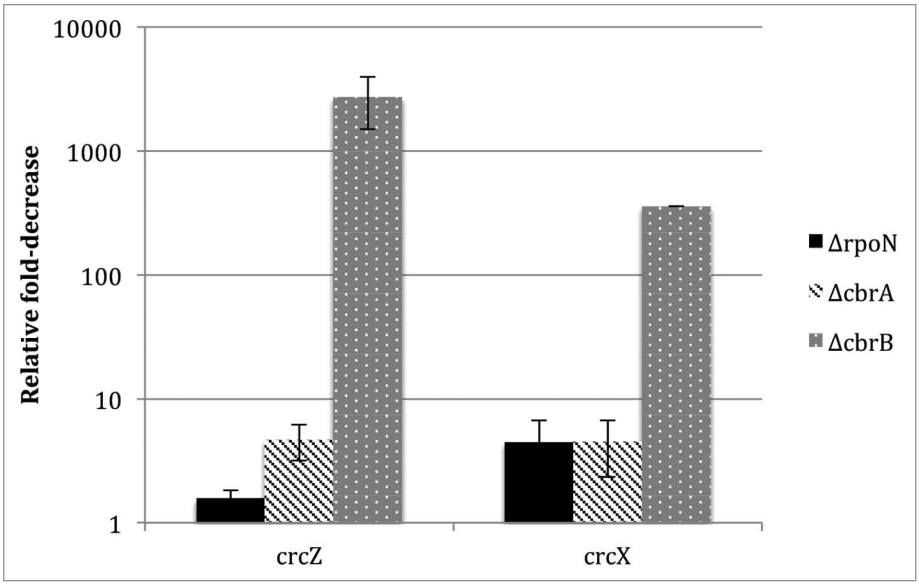

Figure 1. Regulation of *CrcZ* and *CrcX* in *P. syringae*. Relative levels of the *crcZ* and *crcX* ncRNAs, compared to WT, measured by quantitative RT-PCR, in cells grown overnight in KB medium. The levels of gene expression in each sample were calculated as fold expression ratio using *gyrA* as a gene for normalization. The values are averages of three independent experiments and error bars represent standard deviation.

Crc, controlling catabolite repression. However, in contrast to *P. aeruginosa*, succinate and other organic acids only have a small influence on Crc in catabolite repression in *P. putida*.[7] Hence, the levels of *crcZ* and *crcY* are higher in *P. putida* when grown in succinate or citrate compared with cells grown in LB medium, where Crc-mediated catabolite repression is very strong.[6] The levels of *crcZ* and *crcY* are also higher in stationary phase, where no catabolite repression is observed.[6] Additionally, transcript levels of *crcZ* and *crcY* in *P. putida* are higher at lower temperatures.[8]

P. aeruginosa and *P. putida* do not contain *psr2* homologs and a function has not yet been described for this ncRNA. In this report, we analyze the role of *psr1* and *psr2* in modulating carbon metabolism in *P. syringae* DC3000 and examine their conservation in other sequenced *Pseudomonas* strains. Following the nomenclature of Moreno et al.,[6] we refer to these ncRNAs as *crcZ* (*psr1*) and *crcX* (*psr2*).

Results

CbrA and CbrB regulate expression of crcZ and crcX in *P syringae* DC3000. We previously predicted that *crcZ* (*psr1*; PSPTO_5668), *crcX* (*psr2*; PSPTO_5669) and *crcY* (*psr3*) were regulated by RpoN in *P. syringae*,[3] based on the presence of RpoN-promoter motifs upstream of each gene. Although *crcY* (*psr3*) is also flanked by a likely RpoN promoter, this gene is disrupted by an insertion sequence and is not expressed.[3] To test if *crcZ* (PSPTO_5668) and *crcX* (PSPTO_5669) are regulated by RpoN in *P. syringae* DC3000, we compared expression of both genes in a wild-type strain vs. a mutant in which *rpoN* has been disrupted. Both ncRNAs had slightly reduced levels in the RpoN mutant (**Fig. 1**), suggesting at least partial regulation by RpoN in *P. syringae* DC3000, but expression was not completely eliminated. Therefore, we examined whether additional transcriptional start points for *crcZ* and *crcX* could be detected in the RpoN mutant. Our results indicate that for both *crcZ* and *crcX*, alternative transcriptional start points can be detected in the RpoN mutant (data not shown). These transcriptional start points are 2–4 bases downstream of the transcriptional start points detected in the wild-type strain. This result suggests that transcripts for *crcZ* and *crcX* arise from two promoters, one recognized by RpoN and the other by a different sigma factor. It has been shown that *crcZ* in *P. aeruginosa* and *crcZ* and *crcY* in *P. putida* are regulated by RpoN,[5,6] but a second promoter was not reported in these species.

In *P. aeruginosa* and *P. putida*, expression of *crcZ* is activated by the CbrA/CbrB two-component sensor-regulator system.[5,6] However, in *P. putida* ,activation of *crcY* is thought to use a different transcriptional regulator.[6] To investigate the role of CbrAB in expression of *crcZ* and *crcX* in *P. syringae* DC3000, we used qRT-PCR to compare RNA levels in wild-type *P. syringae* DC3000 strain with those lacking *cbrB* or *cbrA*. In cells grown in KB medium, inactivation of the *cbrB* gene significantly reduced expression of both *crcZ* and *crcX* (**Fig. 1**). Additionally, inactivation of *cbrA* also reduced expression of both *crcZ* and *crcX*. The change was not as dramatic as that observed with the *cbrB* mutant, suggesting CbrB may interact with another histidine kinase, allowing for transcription in the absence of CbrA.

Expression of CrcZ and CrcX are influenced by the carbon source. The levels of *crcZ* in *P. aeruginosa* vary according to the carbon source being used and correlate with the relief of catabolite repression.[5] To analyze the influence of carbon source on mRNA levels of *crcZ* and *crcX* in *P. syringae* DC3000, we performed qRT-PCR to evaluate the relative levels of *crcZ* and *crcX* in cells grown in a minimal medium supplemented with various carbon sources. We found that compared with mRNA levels in LM medium at mid-exponential phase, increased levels of *crcZ* and *crcX* were observed in cells grown in fructose, glucose and mannitol, with very high levels being observed when fructose was used as the sole carbon source (**Fig. 2A**). mRNA levels for *crcZ* and *crcX* were also elevated when grown with the organic acid citrate as a sole carbon source. The relative abundance of *crcZ* vs. *crcX* also varies depending on the medium. In LM mid-exponential cultures and in succinate there is more *crcZ* than *crcX,* but when cells are grown in conditions that induce expression of the ncRNAs (fructose, mannitol, glucose, citrate), which are present in approximately the same ratio (**Fig. 2B**). A similar shift was reported in *P. putida*, where *crcZ* is eight times more abundant than *crcY* in LB-mid-log cells, but in non-repressing conditions

(LB medium at stationary phase, or M9 medium supplemented with either succinate or citrate), the ratio decreases. In *P. putida*, levels of *crcZ* and *crcY* are high when grown in succinate (less preferred carbon source) and *crcZ* and *crcY* are lower in *P. syringae* when grown in succinate (a good carbon source). In contrast to what is observed in *P. putida, crcZ* is much more abundant than *crcX* in *P. syringae* when grown in succinate (**Fig. 2B**).

CrcZ and CrcX influence growth of *P. syringae*. Inactivation of *crcZ* in *P. aeruginosa*, or *crcZ* and *crcY* in *P. putida* leads to reduced growth in non-preferred carbon sources.[5,6] For *P. syringae*, deletion of both *crcZ* and *crcX* was required to obtain a difference in growth in mannitol and fructose (**Fig. 3**), suggesting that *crcZ* and *crcX* are functionally redundant in DC3000. The wild-type and mutant strains grew to similar levels with succinate as the sole carbon source (**Fig. 3**). Decreased growth was also observed when citrate (a preferred carbon source for *P. aeruginosa*) was used as the sole carbon source. This might indicate that in *P. syringae* DC3000, the organic acid citrate is a non-preferred carbon source.

Two predicted mRNA targets for Crc in *P. syringae* DC3000 are the L-arabinose transporter permease protein (PSPTO_2640) and a myo-inositol 2-dehydrogenase (PSPTO_3494).[9] Therefore, we tested whether deletion of *crcZ* and *crcX* influenced the growth rate of DC3000 cultured using these substrates as sole carbon sources. Reduced growth was observed for the double mutant when grown with either arabinose or myo-inositol (**Fig. 3**).

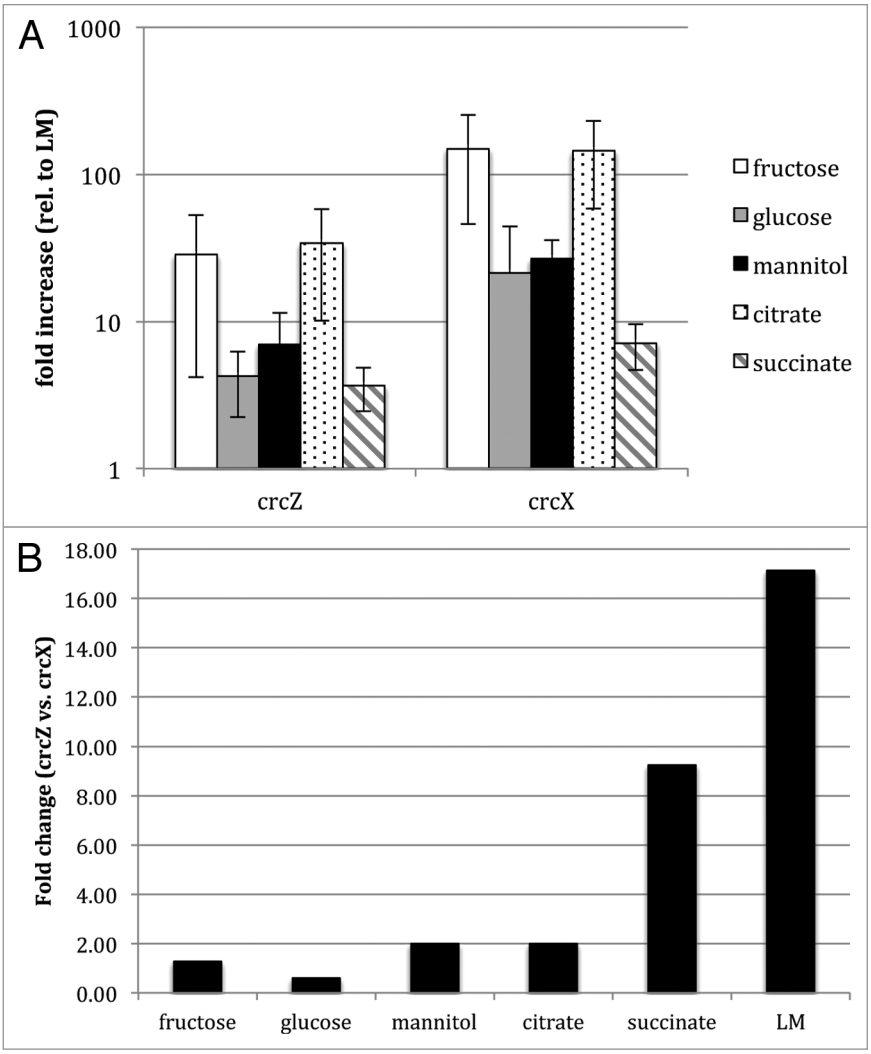

Figure 2. (**A**) Effect of carbon source on levels of *crcZ* and *crcX*. The values shown correspond to the ratios of the RNA levels observed for each condition relative to those observed in cells growing exponentially in LB medium as reference. The standard error is indicated. (**B**) Comparison of the abundance of *crcZ* relative to that of *crcX* under the specified growth conditions, determined by real-time RT-PCR. RNA samples were the same as those used in the assays presented in **A**.

Sequence similarity and secondary structure of CrcX,Y,Z homologs in *P. syringae*. Since *crcY* is disrupted in DC3000, we were interested in addressing two important questions: (1) what is the prevalence of the Crc family of ncRNAs in other sequenced *P. syringae* pathovars and (2) are there any *P. syringae* strains in which *crcY* was disrupted by insertion sequences, as in DC3000? Since exact coordinates for the *crc* ncRNAs are now known and also a large number of pseudomonas genomes have been sequenced, we are able to address these questions. Using the mapped transcriptional start sites, mapped 3'-ends and transcriptional activity profiles[3] we determined the genomic boundaries for *crcZ, crcX*, and *crcY* in DC3000. Because *crcY* is disrupted by an insertion element, the two partial sequences were spliced together to form one continuous "virtual" ncRNA. This information was then used to infer the genomic boundaries for *crcZ*, *crcX* and *crcY* in *P. syringae* strains B728a and 1448A (**Table 1**). Putative transcript sizes varied from 342–368 nt. A consensus structure generated using LocARNA exhibits four to five conserved regions containing CA motifs (**Fig. 4**). The nine ncRNAs are highly conserved, but also exhibit several variable regions. For comparison, the alignments for *crcZ* (*psr1*), *crcX* (*psr2*) and *crcY* (*psr3*) from DC3000, B728a and 1448A were performed separately (see **Figs. S1–S3**). *crcZ* (*psr1*) and *crcX* (*psr2*) contain five conserved "CA" regions, whereas *crcY* (*psr3*) contains four "CA" regions.

Construction of a CrcZ,X,Y covariance model. Using the nine sequences from DC3000, B728a and 1448A, a co-variance model (CM) was constructed using the Infernal toolset. The model was used to search all fully sequenced and draft genomes belonging to the Pseudomonadaceae family to locate *crc* candidates that may have been overlooked in the earlier CMFinder search that relied on the gamma-150 motif.[4] The results of the CMsearch are shown in **Table 2** and **Table S3**. The scans retrieved all of the candidates using an earlier model based on the gamma-150 motif,[4] as well as identified additional *crc* candidates. All

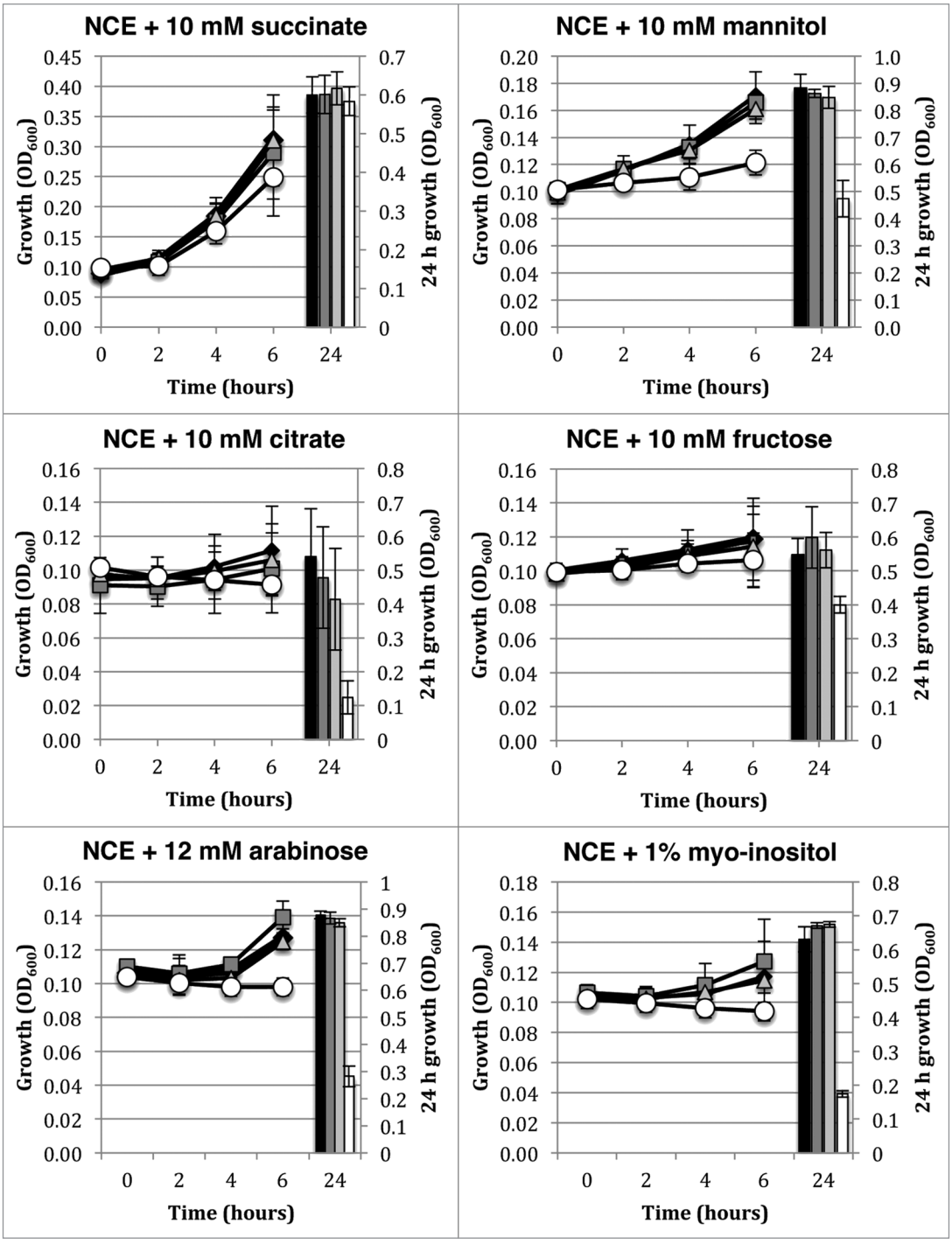

Figure 3. Effect of carbon source on growth of *P. syringae crcZ* and *crcX* mutants. Growth of wild-type DC3000 (solid black diamonds and black bar), ΔcrcZ (dark gray squares and dark gray bar), ΔcrcX (light gray triangles and light gray bar) and ΔcrcZX (white circles and white bar) in NCE supplemented with 10 mM succinate, 10 mM mannitol, 10 mM citrate, 12 mM arabinose or 1% myo-inositol. Samples for growth analyses were harvested at four time points (2, 4, 6 and 24 h).

pseudomonads, except one, had at least one *crc* candidate ncRNA (**Table 2** and **Table S3**). The exception, *P. geniculate* was isolated from river water, but its lineage is currently in question[10] and it is listed as an unclassified Pseudomonas in the NCBI taxonomy browser. Therefore, it is not surprising that the CMSearch did not find a *crc* candidate in this organism. Only one *crc* candidate ncRNA was found in all *P. aeruginosa* and *P. mendocina* strains. A single candidate *crc* ncRNA was found in *P. stutzeri* strain SDM, Pseudomonas 2_1_26, Pseudomonas S9 and *P. psychrotolerans* L19. Two *crc* candidates were found in other pseudomonads such as *P. fluorescens*, *P. stutzeri* and *P. putida* strains (**Table 2 and Table S3**).

Using this model, multiple *crc* candidates were found in all *P. syringae* strains. The model detected at least three *crc* candidates in the *P. syringae* strains, with the exception of *P. syringae pv morsprunorum* M302280PT (two candidates). *P. syringae* strains, which potentially have more than three *crc* candidate ncRNAs, include *P. syringae* pv *japonica* M301072PT (5; two of which contain seven base pair disruptions), *P. syringae* pv *oryzae* (4) and *P. syringae* pv *pisi* (5; one of which contains a seven base pair disruption) and *P. syringae* pv *mori* 301020 (4). As for the tomato pathovars, DC3000 appears to be the only strain in which one of the *crc* ncRNAs (*crcY*) has been disrupted by an insertion.

The gamma-150 motif was also reported in *Azotobacter vinelanii*.[4] To determine if our model yielded similar results, the new model was used to search closely related strains from the Pseudomonadaceae family (Azotobacter, Cellvibrio, Moraxella, Psychrobacter and Acinetobacter). Other than the two ncRNA candidates previously reported for *Azotobacter vinelanii*, we failed to detect *crcZXY* ncRNAs in these strains. Therefore, the Crc family of ncRNAs seems to be unique to the Pseudomonads and Azotobacter strains. The Azotobacter case is consistent with suggestions that *A. vinelandii* may in fact be a Pseudomonad,[11] based on genome similarity. In total, our model detected 237 Crc family ncRNA candidates.

Clustering of CrcZ,X,Y candidates. The Crc ncRNA candidates identified in the closed genomes were clustered using RNAcluster to ascertain the structural relationship between the candidates. The resulting dendogram is shown in **Figure S4**. The known *crcZ* candidates (highlighted in blue) cluster together, as well as the previously reported *crcY* ncRNAs (highlighted in green) and *crcX* ncRNAs (highlighted in red). To further investigate the relationship of the *crc* candidates, the Crc ncRNA candidates identified in the *P. syringae* genomes (closed and draft genomes) were clustered separately (**Fig. S5**). Overall, 105 of 107 sequences cluster into three main families.

Deciphering the relationship of the crc ncRNA candidates. *crcZ.* To evaluate the differences between *crc* ncRNA candidates, we examined their genomic contexts and classified each ncRNA based on its conserved genetic linkage. For *crcZ*, we first identified strains containing homologs for PcnB (PSPTO_0963) and PSPTO_0964. Any *crc* ncRNA candidates located between the PSPTO_0963 and PSPTO_0964 homologs were considered to represent members of the *crcZ* subfamily. Ninty-six CMsearch candidates shared this genomic arrangement (**Table S3**). Nine other CMsearch candidates were located either adjacent to a PSPTO_0964 homolog or a PSPTO_0963 homolog. In these cases, complete conservation is difficult to determine since the positions of the hits within the contigs does not allow for sufficient context and, therefore, characterizing them as *crcZ*-like is less certain. Overall, with the exception of *P. geniculate* (which contains no instance of *crcZ*), all of Pseudomonas strains sequenced to date (103/104) contain at least one *crc* ncRNA candidate (*crcZ)* adjacent to *pcnB*. *P. fluva* contained two ncRNA candidates with this particular genomic arrangement, yielding a total of 105 *crcZ*-like candidates.

crcX. To evaluate the distribution and conservation of *crcX*, we identified strains containing PSPTO_1621 and PSPTO_1622 homologs and compared the locations of the homologs with the coordinates from the CMSearch. Those *crc* ncRNA candidates located between a PSPTO_1621 and PSPTO_1622 homologs were considered to represent the *crcX* class. Of the 26 putative *crcX* ncRNAs identified, all were found to be exclusive to *P. syringae* strains (**Table S3**). In addition, 15 other CMsearch candidates were located either upstream of PSPTO_1621 or PSPTO_1622 homologs. As with *crcZ*, some of these may be bona fide *crcX* instances, but their status is provisional until their corresponding genome sequences can be closed. Taken together, all except one of the *P. syringae* strains (37 out of 38) contained a *crc* ncRNA candidate in proximity to PSPTO_1621 or PSPTO_1622 homologs. The single exception was *P. syringae pv morsprunorum* M302280PT, which does not appear to contain a *crcX* ncRNA. All together, the data suggest that the ncRNA *crcX* is exclusive to *P. syringae* strains.

Table 1. Pseudomonas syringae crcZ, crcX and crcY coordinates

P. syringae strain	ncRNA	5'end	3'end	Length
DC3000	*crcZ*	1046951c	1046608c	343
	crcX	1778751c	1778383c	368
	crcY	3047265c	3044933c	361[a]
B728a	*crcZ*	941123c	940780c	343
	crcX	4476099	4476467	368
	crcY	2865015c	2864663c	352
1448A	*crcZ*	1024980c	1024637c	343
	crcX	1738895c	1738527c	368
	crcY	3034385c	3034033c	352

[a]For *P. syringae* pv tomato strain DC3000 *crcY*, the length was determined by omitting the insertion sequence and splicing the two fragments together. c, Expression occurs on the negative, complementary, strand.

crcY. In DC3000, PSPTO_2740 and PSPTO_2741 are transposon-related sequences that disrupt *crcY*, but are not found in *P. syringae* strains B728a or 1448A. To classify putative *crcY* candidates, we instead used PSPTO_2742 and PSPTO_2739 as context markers and identified strains containing their homologs. We then asked if a candidate ncRNA was located between the homologs or was located in close proximity (< 400 bps) of these homologs. Seventy-six CMsearch candidates satisfied these criteria and represent putative *crcY* ncRNAs. Interestingly, this ncRNA appears to be present in all Pseudomonas strains, except for *P. aeruginosa* and *P. mendocina* strains and *P. syringae* pv *aceris* M302273PT.

Several CMsearch candidates were located in genomic locations that could not be classified using the BLAST analyses described above. Some occurred in strains that have more than three *crc* ncRNA candidates (*P. syringae japonica, P. syringae mori, P. syringae oryzae* and *P. syringae pisi)*. In these pathovars, we found that the CMsearch candidates were located on extremely short contigs (< 500 bps) and, in most cases, the contig is smaller than the co-variance model itself.

Regulatory features of the crc candidate ncRNAs. The upstream regions of the 96 *crcZ* candidates were aligned to identify

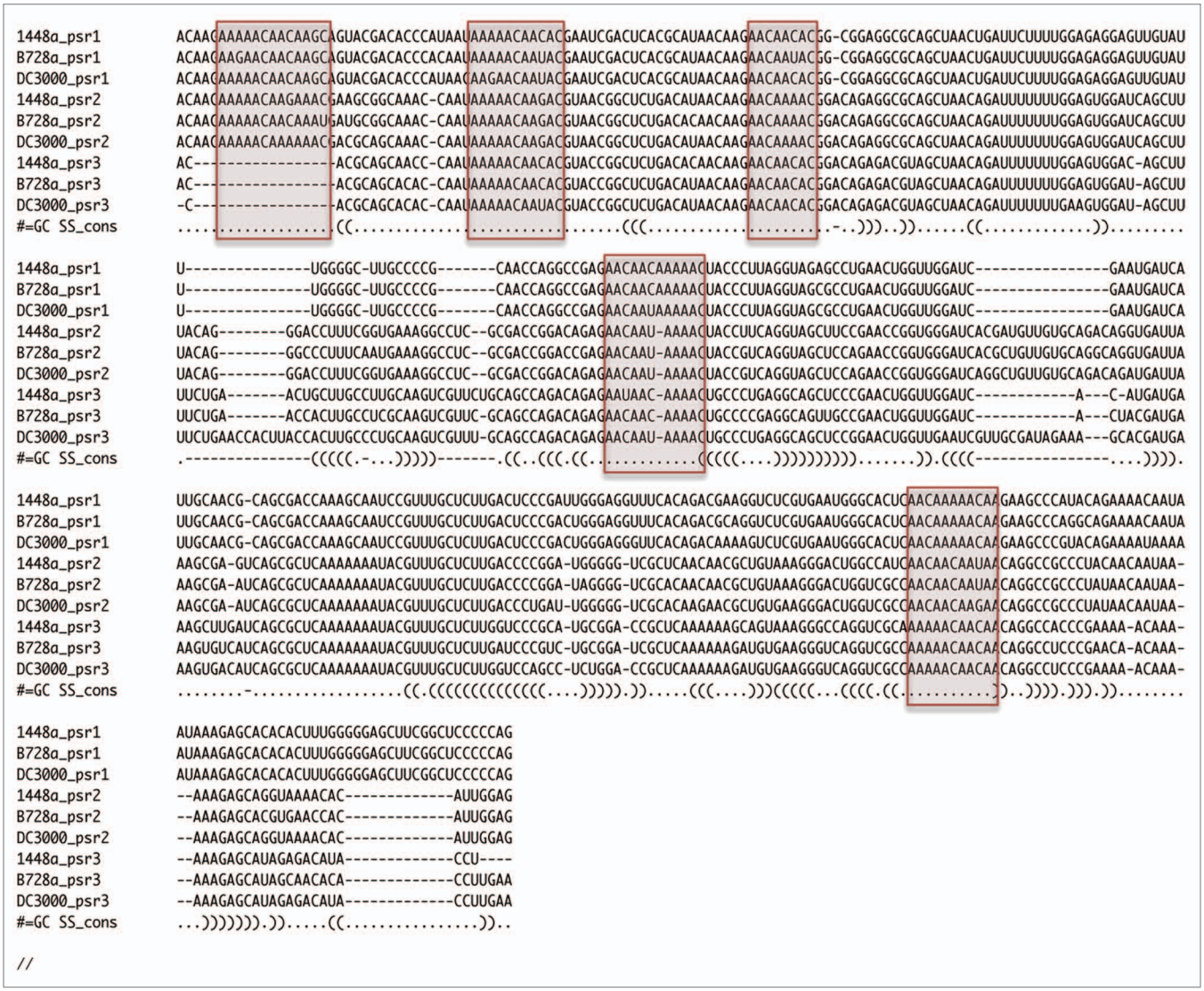

Figure 4. LocARNA alignment of the nine *crcZ,X,Y* (*psr1,2,3*) genes in *Pseudomonas syringae* pv *tomato* str. DC3000, *Pseudomonas syringae* pv *pha-seolicila* str. 1448A and *Pseudomonas syringae* pv *syringae* str. B728a. # = GC SS_cons represents the consensus structure. In the consensus sequence, matching nested parentheses are indicated by "()." The symbol "." indicates unpaired regions. Sequences containing "CA" motifs are highlighted with pink boxes.

potential conserved regulatory features. The analysis revealed the existence of several highly conserved motifs (**Fig. S6**). One motif resembles the RpoN promoter region, which has a highly characteristic -24/-12-regions with the consensus sequence: 5'-YTG**G**CACG-N4-TTG**C**W-3', (the bold G and C at positions -24 and -12 relative to the start of transcription).[12] This motif is similar to the RpoN-binding site for *P. fluorescens* and *P. putida* and its presence is consistent with reports that RpoN regulates the expression of *crcZ*.[5,6] Further upstream, we also identified a sequence similar to the published consensus CbrB-binding site for *P. aeruginosa* (cTGTTACc-$N_{3/12}$ -cGTAACAg)[13] (**Figs. S6 and S7**), suggesting that all *crcZ* ncRNAs are regulated by the CbrA/CbrB two-component system.

Our data also suggests that *crcX* in DC3000 is regulated by CbrB (**Fig. 1**). To explore this further, regions upstream of *crcX* candidates were aligned (**Figs. S7 and S8**). As expected, a putative RpoN promoter region was present. We also found one-half of the predicted CbrB-binding site at -154 to -147 (TGTTACC). A motif similar to the other half of the predicted CbrB-binding site (GTAACAC) was located 29 bases away from the other half of the binding site. This interval is larger than the 3-bp or 12-bp spacing reported for some CbrB-binding sites[13] but is similar to the spacing observed upstream of *crcY* in *P. putida*.[6] However, it has been reported that inactivation of CbrB has little effect on the expression of *crcY* in that organism.[6] The same palindrome with a 29 bp spacer occurs upstream of putative *crcY* candidate ncRNAs (**Figs. S7 and S9**).

Discussion

In this study, we find that the Crc ncRNA family is common throughout the genus *Pseudomonas*. As with the *rsmX* ncRNAs,

Table 2. Crc ncRNA candidates identified in fully sequenced Pseudomonas genomes using CMsearch

Strain	Accession	Score	5'-end	3'-end
Acinetobacter baumannii AYE	CU459140.1	15.73	78400	78422
Azotobacter vinelandii DJ	CP001157.1	94.02	4303297	4303638
Azotobacter vinelandii DJ	CP001157.1	176.76	452815c	452475c
Pseudomonas aeruginosa LESB58	FM209186.1	145.66	5642408	5642759
Pseudomonas aeruginosa M18	CP002496.1	143.25	5361488	5361839
Pseudomonas aeruginosa NCGM2 S1	AP012280.1	145.03	888676c	888325c
Pseudomonas aeruginosa PA7	CP000744.1	136.47	5610663	5611013
Pseudomonas aeruginosa UCBPP-PA14	CP000438.1	144.16	5580520	5580871
Pseudomonas aeruginosa	AE004091.2	145.4	5308589	5308940
Pseudomonas brassicacearum NFM421	CP002585.1	216.88	5835658	5836004
Pseudomonas brassicacearum NFM421	CP002585.1	244.44	2260512c	2260075c
Pseudomonas entomophila L48	CT573326.1	191.09	5029853	5030198
Pseudomonas entomophila L48	CT573326.1	191.06	3150228	3150563
Pseudomonas fluorescens F113	CP003150.1	243.42	4575452	4575888
Pseudomonas fluorescens F113	CP003150.1	217.08	5792772	5793163
Pseudomonas fluorescens Pf0 1	CP000094.2	237.33	4135995	4136343
Pseudomonas fluorescens Pf0 1	CP000094.2	220.71	5425228	5425569
Pseudomonas fluorescens Pf-5	CP000076.1	221.77	4546202	4546554
Pseudomonas fluorescens Pf-5	CP000076.1	211.49	6040962	6041305
Pseudomonas fluorescens SBW25	AM181176.4	217.28	5747060	5747403
Pseudomonas fluorescens SBW25	AM181176.4	207.9	4288010	4288357
Pseudomonas fulva 12 X	CP002727.1	127	4826693	4826974
Pseudomonas fulva 12 X	CP002727.1	90.4	1039803c	1039482c
Pseudomonas mendocina NK 01	CP002620.1	160.54	4286402	4286744
Pseudomonas mendocina ymp	CP000680.1	158.25	3959213	3959556
Pseudomonas putida BIRD 1	CP002290.1	211.49	4910564	4910909
Pseudomonas putida BIRD 1	CP002290.1	184.83	2566512c	2566177c
Pseudomonas putida F1	CP000712.1	214.87	5102227	5102575
Pseudomonas putida F1	CP000712.1	190.89	2543815c	2543483c
Pseudomonas putida GB 1	CP000926.1	214.82	5246762	5247112
Pseudomonas putida GB 1	CP000926.1	190.63	2668606c	2668277c
Pseudomonas putida KT2440	AE015451.1	213.48	5338277	5338622
Pseudomonas putida KT2440	AE015451.1	198.23	4013244	4013576
Pseudomonas putida S16	CP002870.1	209.22	5140236	5140582
Pseudomonas putida S16	CP002870.1	195.87	3401961	3402289
Pseudomonas putida W619	CP000949.1	214.69	814356c	814007c
Pseudomonas putida W619	CP000949.1	202.25	2173887c	2173555c
Pseudomonas stutzeri A1501	CP000304.1	174.07	2072801	2073136
Pseudomonas stutzeri A1501	CP000304.1	133.16	3565446	3565788
Pseudomonas stutzeri ATCC 17588 LMG 11199	CP002881.1	165.37	1910377	1910712
Pseudomonas stutzeri ATCC 17588 LMG 11199	CP002881.1	132.98	3575287	3575629
Pseudomonas stutzeri DSM 4166	CP002622.1	173.57	2058333	2058668
Pseudomonas stutzeri DSM 4166	CP002622.1	134.22	3663966	3664308
Pseudomonas syringae phaseolicola 1448A	CP000058.1	340.83	1738895c	1738527c
Pseudomonas syringae phaseolicola 1448A	CP000058.1	309.67	3034385c	3034033c
Pseudomonas syringae phaseolicola 1448A	CP000058.1	294.01	1024980c	1024622c

Table 2. Crc ncRNA candidates identified in fully sequenced Pseudomonas genomes using CMsearch

Pseudomonas syringae B728a	CP000075.1	333.11	4476099	4476467
Pseudomonas syringae B728a	CP000075.1	319.24	2865015c	2864663c
Pseudomonas syringae B728a	CP000075.1	291.23	941123c	940765c
Pseudomonas syringae tomato DC3000	AE016853.1	337.19	1778751c	1778383c
Pseudomonas syringae tomato DC3000	AE016853.1	293.85	1046951c	1046593c
Pseudomonas syringae tomato DC3000	AE016853.1	193.29	3045294c	3044933c
Pseudomonas syringae tomato DC3000	AE016853.1	107.52	3047265c	3047005c

P. syringae strains contain multiple copies of *crc* ncRNAs.[14] Paralogous ncRNAs can act redundantly or additively within the bacteria cell.[15-19] The growth analyses presented here suggest that CrcZ and CrcX act redundantly in at least one pathway. However, it is possible that they participate in other pathways as well, where they perform distinct functions.

crcZ is highly conserved and appears to be the only *crc* ncRNA present in the *P. aeruginosa* strains, and others that are able to cause human disease such as *P. mendocina*. Although some members of the Pseudomonacaeae such as Acinetobacter and Morexella contain PcnB homologs, our model did not detect candidate ncRNAs in these cases. *Acinetobacter baylyi* does have a Crc protein involved in catabolite repression control and aromatic compound catabolism and Crc is reported to act post-transcriptionally as in the Pseudomonads,[20] but in contrast to the Pseudomonads, it displays a strong effect on transcript stability. It is possible that in Acinetobacter, the levels of Crc are modulated at the transcript level and there is no requirement for ncRNAs. Alternatively, Acinetobacter strains may have ncRNAs distinct from *crcX,Y* and *Z* that regulate the levels of Crc protein. Of the 104 bacteria containing CrcY/Z/X candidates, only one (*Pseudomonas aeruginosa* strain 152504_uid62725) has no PSPTO_0079 homolog (data not shown). However, the absence of a Crc ortholog may be an artifact, since a "closed" genomic sequence is not yet available for this strain. Overall, our results show that *crcYZX* ncRNAs are nearly always accompanied by a Crc ortholog.

One of the most exciting results from this study is that our analysis revealed that CrcX is only found in the *P. syringae* strains. This finding is important for several reasons. One is that this ncRNA or its flanking regions may be useful as a diagnostic tool to identify members of this group of plant pathogens. Second, is that the presence of this region in *P. syringae* strains may be linked to species-specific traits found only in *syringae* strains. *P. syringae* is physiologically different from non-pathogenic Pseudomonads (using the LOPAT scheme which evaluates oxidase production, arginine dihydrolase, activity, aerobic growth and carbon source utilization). Experimental analyses of nutrient assimilation by pseudomonads indicate that plant pathogenic *P. syringae* strains assimilate a restricted range of nutrient sources compared with other pseudomonads[21] and is physiologically specialized for growth using the most abundant amino acids in plant tissues and on the plant surface. In contrast, the *P. syringae* lineage lacks some metabolic reactions that are conserved in other Pseudomonads.[22] CrcX may therefore be involved in the precise control of the limited pathways used for primary metabolism.

Although several reports have provided insight into the biochemistry and genetics of metabolism in pseudomonads, relatively little is known about carbon catabolism in *P. syringae*. Catabolite repression control (CRC) is an important global control system in Pseudomonas that fine tunes metabolism to optimize growth in a variety of different environments.[9] Crc is involved in several Pseudomonas species in catabolite repression of the branched-chain keto acid dehydrogenase[23] and of alkane degradation,[7] as well as of a number of enzymes involved in aromatic compound degradation.[24] In addition to modulating metabolism, in *P. aeruginosa*, Crc influences susceptibility to antibiotics, expression of Type III secretion, expression of quorum sensing-regulated virulence factors, such as pyocyanin[25] and potentially other virulence functions.[26] Sugars such as glucose, sucrose and fructose are known to be inducers of the *P. syringae* TTSS genes, whereas tricarboxylic acids (TCA) intermediates can suppress T3SS in vitro. Fructose and citrate utilization pathways used by *P. syringae* are upregulated when cells are exposed to apoplast extracts.[21] Therefore, during plant infection, we hypothesize that when T3SS is active, the expression of these ncRNAs may support utilization of these carbon sources.

In contrast to the Rsm system where there are several RNA binding proteins, the presence of only one Crc gene in the pseudomonads implies that all of the Crc ncRNAs function through a single protein. Bioinformatic analysis of predicted Crc binding sites in Pseudomonas genomes[9] suggests that some targets are genus-wide and related to central metabolism, whereas others are predicted to be species-specific or unique to particular strains. Predictions for DC3000 include *algP* (alginate metabolism), PSPTO_3494 (myo-inositol 2-dehydrogenase), proteins involved in chemotaxis and several transcriptional regulators. Our data implicate CrcZ and CrcX in myo-inositol utilization. Studies are underway to investigate the role of these ncRNAs in other pathways.

Materials and Methods

Bacterial growth conditions. For routine growth, *Pseudomonas syringae* pv *tomato* DC3000 was maintained at 28°C on King's broth (KB)[27] agar plates. For growth studies with various carbon sources, bacterial cells were grown in LM at 28°C with shaking overnight. The cells were sub-cultured to an OD_{600} of 0.1 in LM or No-carbon-E-minimal medium (NCE)[28] supplemented with fructose (10 mM), glucose (10 mM), mannitol (10 mM), citrate (10 mM), succinate (10 mM), myo-inositol (1%) or arabinose (12 mM) as the carbon source.

RNA isolation. Total RNA was isolated from cultured cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol using the on-column DNase treatment with the exception that lysozyme was used at a concentration of 5 mg/ml. Isolated RNA was further treated twice with two units of DNase (Ambion) to eliminate DNA contamination. RNA was purified from the DNase mixture using MinElute column (Qiagen).

5' and 3' RACE. 5' RACE assays were performed using the 5' RACE System for the Rapid Amplification of cDNA Ends, v2.0 kit (Invitrogen) as described by Moll et al.[14] Five hundred nanograms to 1 μg of isolated RNA were used in each reaction and the procedure was performed following the manufacturer's protocol. Following amplification with a gene-specific primer (GSP2), PCR products were separated on a 2% agarose gel and bands of interest were excised, gel-eluted (Zymoclean Gel DNA Recovery kit, Zymo Research) and sequenced (Genomics facility, Life Sciences Core Laboratories, Cornell University). Oligonucleotides used for reverse transcription and PCR are listed in **Table S1**.

3' RACE was performed as described by Moll et al.[14] This protocol was adapted from Argaman et al.[29] Briefly, 1 μg of RNA was mixed with 100 pmol of RNA adaptor (5'- phosphate-UUCACUGUUCUUAGCGGCCGCAUGCUC-idT-3'), heat-denatured at 95°C for 5 min, then quick-chilled on ice. The adaptor was ligated at 17°C for 12 h in the presence of 40 units of T4 RNA ligase (New England Biolabs) and 40 units of RNase OUT (Ambion) in a buffer containing 50 mM TRIS-HCl (pH 7.9), 10 mM $MgCl_2$, 4 mM DTT, 150 μM ATP and 10% DMSO. The ligated RNA product was purified from the reaction using the RNA Clean and Concentrator -5 kit (Zymo Research) and reverse-transcribed using 20 pmol of a single primer complementary to the RNA adaptor (A1). Reverse transcription was performed using the Thermoscript RT system (Invitrogen) according to the manufacturer's protocol. The products of reverse transcription were amplified using a 2 μl aliquot of the RT reaction, 20 pmol of each gene-specific and adaptor-specific primer (A2) and 1X Ex-Taq Polymerase mix (TaKaRa Bio). Cycling conditions were as follows: 95°C/2 min; 35 cycles of 94°C/30 sec, 57°C/45 sec, 72°C/30 sec; 72°C/10 min. PCR separation, gel extraction, cloning and sequencing were performed as described for 5' RACE. Primers used in 3' RACE analyses are shown in **Table S1**.

Construction of the RpoN mutant. Construction of the DC3000 *RpoN* mutant was performed by PCR amplification of an internal sequence of *rpoN*, corresponding to nucleotides 100–700 of the coding sequence, from the DC3000 genome and cloned into pKnockout-Ω.[30] The resulting plasmid was introduced into DC3000 via electroporation. Since pKnockout cannot replicate in DC3000, single-crossover integrants were selected for resistance to spectinomycin. Orientation of integration was determined by PCR.

Construction of *cbrB* and *cbrA* mutants. Flanking regions of approximately 800 bps upstream and downstream of *cbrA* were amplified using the oligomers ΔcbrA1fwd and ΔcbrA1rev, or ΔcbrA2fwd and ΔcbrA2rev, respectively. ΔcbrA1fwd and ΔcbrA2rev contain an external *Xba*I site and ΔcbrA1rev and ΔcbrA2fwd contain an *Nde*I site to allow ligation of the two flanking DNA fragments. The fragments were amplified using the Expand high-fidelity PCR system (Roche). The PCR fragments were gel purified with the Zymoclean gel DNA recovery system. The two fragments, ΔcbrA1 and ΔcbrA2 or ΔcbrA1 and ΔcbrA2, were digested with *Nde*I, joined by ligation and then amplified using the primers ΔcbrA1fwd and ΔcbrA2rev. The product was gel purified, digested with *Xba*I and cloned into pK18mobsacB using the *Xba*I restriction site. For construction of the *cbrB* mutant, 1.0 kb regions directly upstream and downstream of *cbrB* were amplified by PCR. Gel purified PCR fragments were joined by SOEing in a second PCR amplification with primers containing *XbaI* restriction sites. The product was gel purified using Gel DNA Recovery Kit (Zymo Research), digested *XbaI* and cloned into pK18mobsacB cut with the same restriction enzyme.

The plasmids were transformed into DC3000 by electroporation followed by selection for integration on LM containing 50 μg/ml kanamycin. Colonies then transferred to 10% sucrose medium to select for crossover events that resulted in the loss of the *sacB* gene. Sucrose-resistant colonies were screened by PCR and positive clones (those containing the deletion) were confirmed by sequencing.

Construction of *crcZ, crcX* and *crcZ/X* double mutant. To make the ΔcrcZ mutant strain, a deletion was created using a pK18mobsacB plasmid.[31] pK18mobsacB/ΔcrcZ was created by PCR amplification of DNA fragments of approximately 1.0 kb upstream and downstream of the ncRNA. Gel purified PCR fragments were joined by SOEing in a second PCR amplification with primers containing *Hind*III and *BamH*I restriction sites. The product was gel purified using Gel DNA Recovery Kit (Zymo Research), digested with *Hind*III and *BamH*I and cloned into pK18mobsacB cut with the same restriction enzymes. Similarly, pK18mobsacB/Δ*crcX* was created by PCR amplification of DNA fragments of approximately 1.0 kb that flank *crcX*. Gel purified PCR fragments were joined by a second PCR amplification with primers containing *Hind*III and *BamH*I restriction sites. The product was gel purified, digested with *Hind*III and *BamH*I and cloned into *Hind*III/*BamH*I digested pK18mobsacB.

The pK18mobsacB deletion constructs were confirmed by sequencing at Cornell University Life Sciences Core Laboratories Center before introducing into DC3000 via electroporation. Integration events were selected on KB medium containing 50 μg/ml kanamycin and then transferred to 10% sucrose medium to select for crossover events that resulted in the loss of the *sacB* gene. Sucrose-resistant colonies were screened by PCR and clones containing the deletion were confirmed by sequencing. To construct the *crcZX* double mutant, the pK18mobsacB/Δ*crcX* was introduced into the Δ*crcZ* mutant strain.

Quantitative RT-PCR. Cells were gown at 28°C in flasks in either LM or in NCE medium supplemented with the indicated carbon source. At mid-exponential phase or at stationary phase, samples were collected, harvested by centrifugation and immediately frozen at -80°C. RNA was prepared with the RNeasy Kit (Qiagen) as described above.

Total RNA (100 ng) was reverse transcribed in a thermocycler using the qScript cDNA Supermix (Quanta Biosciences)

according to the manufacturer's instructions. qPCR was performed with 10 mg of cDNA using IQ SYBR green Supermix (Bio-Rad) on a iQ5 multicolor real-time detection system (BioRad). DNA contamination and the formation of primer dimers were assessed by using controls lacking reverse transcriptase and template, respectively. The production of non-specific products was determined by the dissociation protocol included in the software provided with the machine. The resulting cycle thresh-old (Ct) values were calculated by the software and analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in **Table S1**. The Ct values of each gene tested were normalized to the Ct values of the housekeeping genes *gyrA*.

Computational analyses. A list of all genomes used in this study and their GenBank and Refseq accession numbers is provided in **Table S2**. The nucleotide sequences of the three *crc* genes from *P. syringae* pv *tomato* DC3000, *P. syringae* pv *syringae* B728a and *P. syringae* pv *phaseolicola* 1448A were manually extracted and assembled into a single FASTA file. Prediction of secondary structure, consensus model building, calibration and searching were performed as described in ref. 14. The following software package versions were used: RNAclust v1.2.5 (www.bioinf.uni-leipzig.de/~kristin/Software/RNAclust/); RNAalifold[32] ViennaRNA v1.8.5 (www.rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi); locarna v1.6.2[33] and infernal v1.0.2.[34] Results from the CM search were filtered to retain matches with E values less than 1e-3. This resulted in a total of 237 CMfinder candidates.

When clustering the *crc* candidates, since some were substantially shorter than *crc* genes used to build the model, we chose to discard all sequences shorter than 280 bases. This resulted in a total of 107 sequences used in the clustering for the closed genomes and 51 sequences for clustering candidates from the *P. syringae* genomes (draft and closed). Nw_display from the Newick-Utils package v1.6[35] was used to render the clustering results as a dendogram.

Syntenic regions were identified based on homology of neighboring genes. Homology was estimated using tblastn from the NCBI Blast+ package version 2.2.26.[36] All of the genomes listed in **Table S2** were used to construct a single database and the following protein sequences were used as the query input: PSPTO_0963, PSPTO_0964, PSPTO_1621, PSPTO_1622, PSPTO_2739 and PSPTO_2742. An e value cutoff of 1e-10 was used.

For each high-quality CM match, a summary was generated listing the match and any high-quality BLAST hit to the query protein sequences that fell within 50,000 bases of the CM match. These summaries were manually curated and given putative assignment as *crcX*, *crcY*, *crcZ* or "unknown" genes.

To discover potential regulatory features upstream of *crc* candidates, 198 bases upstream of the putative *crcZ* genes was extracted and aligned using "clustalw2" from the CLUSTALW package v2.0.12[37] using default parameters. This process was repeated for the putative *crcX* and *crcY* genes. Mview from the MView package v1.52[38] was used to generate a custom colormap.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/23019

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