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# Evolution of *Rhodopseudomonas palustris* to degrade halogenated aromatic compounds involves changes in pathway regulation and enzyme specificity

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**ABSTRACT** Halogenated aromatic compounds are used in a variety of industrial applications but can be harmful to humans and animals when released into the environment. Microorganisms that degrade halogenated aromatic compounds anaerobically have been isolated but the evolutionary path that they may have taken to acquire this ability is not well understood. A strain of the purple nonsulfur bacterium, *Rhodopseudomonas palustris*, RCB100, can use 3-chlorobenzoate (3-CBA) as a carbon source whereas a closely related strain, CGA009, cannot. To reconstruct the evolutionary events that enabled RCB100 to degrade 3-CBA, we isolated an evolved strain derived from CGA009 capable of growing on 3-CBA. Comparative whole-genome sequencing of the evolved strain and RCB100 revealed both strains contained large deletions encompassing *badM*, a transcriptional repressor of genes for anaerobic benzoate degradation. It was previously shown that in strain RCB100, a single nucleotide change in an alicyclic acid coenzyme A ligase gene, named *aliA*, gives rise to a variant AliA enzyme that has high activity with 3-CBA. When the RCB100 *aliA* allele and a deletion in *badM* were introduced into *R. palustris* CGA009, the resulting strain grew on 3-CBA at a similar rate as RCB100. This work provides an example of pathway evolution in which regulatory constraints were overcome to enable the selection of a variant of a promiscuous enzyme with enhanced substrate specificity.

**IMPORTANCE** Biodegradation of man-made compounds often involves the activity of promiscuous enzymes whose native substrate is structurally similar to the man-made compound. Based on the enzymes involved, it is possible to predict what microorganisms are likely involved in biodegradation of anthropogenic compounds. However, there are examples of organisms that contain the required enzyme(s) and yet cannot metabolize these compounds. We found that even when the purple nonsulfur bacterium, *Rhodopseudomonas palustris*, encodes all the enzymes required for degradation of a halogenated aromatic compound, it is unable to metabolize that compound. Using adaptive evolution, we found that a regulatory mutation and a variant of promiscuous enzyme with increased substrate specificity were required. This work provides insight into how an environmental isolate evolved to use a halogenated aromatic compound.

**KEYWORDS** *Rhodopseudomonas palustris*, 3-chlorobenzoate, 4-fluorobenzoate, anaerobic aromatic compound degradation

Halogenated aromatic compounds are widely used in a variety of applications but also have harmful effects on human and animal health (1, 2). Their widespread use has led to their accumulation in the environment, and much research has been done to understand the role microorganisms play in degrading these compounds (2). One such example is the purple nonsulfur bacterium, *Rhodopseudomonas palustris*

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RCB100. *R. palustris* RCB100 has been shown to metabolize 3-chlorobenzoate (3-CBA) during photoheterotrophic growth using the benzoyl-CoA degradation pathway (3). In this pathway, aromatic compounds are first activated by a coenzyme A (CoA) ligase to benzoyl-CoA and then reduced by the enzyme benzoyl-CoA reductase (Fig. 1) (4). In *R. palustris* RCB100, the CoA ligase responsible for activating 3-CBA shows a higher specificity for chlorobenzoates, and this specificity is due to a single nucleotide change in *aliA*, which encodes an alicyclic acid CoA ligase (5). This change results in a variant of AliA with a serine instead of a threonine found in other *R. palustris* strains at amino acid 208 (T208S). AliA$^{T208S}$ has 10-fold more activity with 3-CBA than AliA from *R. palustris* CGA009 (5). After activation by a CoA ligase, 3-chlorobenzoyl-CoA is dearomatized by benzoyl-CoA reductase leading to spontaneous elimination of the halogen and rearomatization to form benzoyl-CoA (6, 7). In addition to 3-chlorobenzoyl-CoA, benzoyl-CoA reductase can dehalogenate other halobenzoyl-CoA substrates like 4-fluorobenzoyl-CoA (Fig. 1) (6–8).

Most strains of *R. palustris*, including the well-known strain CGA009, cannot use 3-CBA as a primary carbon source (9, 10). However, it has been observed that many of these strains can acquire the ability to metabolize 3-CBA within a relatively short period of time (11). This indicates that only one or a few genetic mutations may be necessary to enable 3-CBA metabolism. Presumably, any *R. palustris* strain containing the benzoyl-CoA degradation pathway would be capable of using 3-CBA if the *aliA* allele from RCB100 is introduced. However, introduction of the *aliA*$^{T208S}$ allele in *R. palustris* CGA009 is not sufficient to confer the ability to use 3-CBA as a sole carbon source (5) (Fig. S1). This suggests the presence of another unidentified factor that is required for 3-CBA metabolism in *R. palustris* RCB100.

To determine what this factor may be, *R. palustris* CGA009 was experimentally evolved to grow photoheterotrophically using 3-CBA as a sole source of carbon. Whole-genome sequencing of the evolved strain and *R. palustris* RCB100 led to the discovery of a large deletion that included *badM*, a transcriptional repressor of genes involved in anaerobic benzoate degradation. Deletion of *badM* is sufficient to allow *R. palustris* CGA009 to degrade 3-CBA, and introduction of the *aliA*$^{T208S}$ allele from *R. palustris* RCB100 into *R. palustris* CGA009 Δ*badM* results in a strain with a faster growth rate on 3-CBA. We present a model in which RCB100 initially acquired a deletion that led to constitutive expression of CoA ligases and benzoyl-CoA reductase and then accumulated other mutations that enhanced its ability to metabolize 3-CBA.

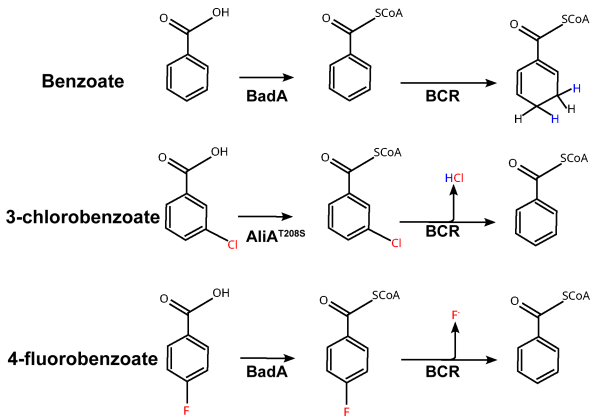


**FIG 1** Proposed pathways for benzoate, 3-CBA, and 4-fluoroacetate degradation in *R. palustris*. Although not shown, the 1,5-dienoyl-CoA formed by benzoyl-CoA reductase (BCR) is further metabolized to acetyl-CoA. Both 3-CBA and 4-flourobenzoate generate benzoyl-CoA after dehalogenation, which is then metabolized as shown in the top reaction.

## RESULTS

### Strains capable of degrading 3-CBA contain large deletions near the bad operon

To understand what other factors may be required to enable *R. palustris* to use 3-CBA, a strain closely related to *R. palustris* RCB100 that is unable to metabolize 3-CBA was identified for use in adaptive evolution. The average nucleotide identity (ANI) of 13 *R. palustris* strains was analyzed with BLASTn. As shown in Fig. 2A, *R. palustris* CGA009 is the most closely related to *R. palustris* RCB100 with an ANI score of 0.99, corresponding to 99% identity between the two genomes. *R. palustris* CGA009 is unable to grow with 3-CBA as a carbon source, and we confirmed that introduction of *aliA*$^{T208S}$ into *R. palustris* CGA009 is not sufficient to enable *R. palustris* CGA009 to grow with 3-CBA as its sole carbon source (Fig. S1) (5).

To select an adapted strain that can grow with 3-CBA as the sole carbon source, CGA009 was grown in a medium with a mixture of benzoate and 3-CBA for several months in the light. After three successive transfers of enrichment cultures into a liquid medium with an increasing ratio of 3-CBA to benzoate, an evolved strain (EVOL-C2) derived from *R. palustris* CGA009 was isolated that was able to grow with 3-CBA as the sole carbon source. Unlike *R. palustris* CGA009, EVOL-C2 can use 3-CBA as a sole source of carbon, although it grew much more slowly than *R. palustris* RCB100 (Fig. 2B; Table 1).

To determine the genetic basis of the evolved phenotype, the genome of EVOL-C2 was sequenced and compared to *R. palustris* CGA009. The sequenced genome of *R. palustris* RCB100 was also compared to *R. palustris* CGA009 (12). Unexpectedly, we found that both EVOL-C2 and *R. palustris* RCB100 contained large deletions near the *bad* operon, which encodes genes required for benzoyl-CoA degradation (Fig. 3A; Table S1). In EVOL-C2, the deleted region (6,521 bp) included partial deletion of *badM* and *hbaA*, and complete deletion of *badL*, *hbaH*, *hbaG*, *hbaF*, and *hbaE*. *R. palustris* RCB100 also contained a similar but significantly larger (10,387 bp) deletion that encompasses *badM* through *hbaC* (Fig. 3A; Table S1). This deletion was the only genetic change that was shared between EVOL-C2 and *R. palustris* RCB100 (Table S1).

The *hba* genes are associated with degradation of 4-hydroxybenzoate (4-HBA). Genes *hbaHGFE* encode a predicted ABC transport system for 4-HBA (4). Like benzoate, 4-HBA is activated by a 4-HBA-CoA ligase encoded by *hbaA*, and *hbaBC* encodes components of 4-HBA-CoA reductase that reductively removes the hydroxyl group from 4-HBA-CoA to yield benzoyl-CoA. Deletion of these genes would be expected to impact the ability of EVOL-C2 and *R. palustris* RCB100 to grow with 4-HBA as the sole carbon source. As shown in Fig. 3B and Table 1, EVOL-C2 grew more slowly on 4-HBA compared to its parent strain, *R. palustris* CGA009, and *R. palustris* RCB100 was unable to grow with 4-HBA as a sole carbon source, confirming the presence of deletions that disrupt 4-HBA metabolism.

### Deletion of the transcriptional repressor *badM* in *R. palustris* CGA009 is sufficient to enable degradation of 3-CBA

Two genes that play a role in the regulation of the *bad* operon, *badL* and *badM*, were also deleted in EVOL-C2 and *R. palustris* RCB100 (Fig. 3A). The *bad* operon encodes the benzoyl-CoA ligase, BadA; a ferredoxin, BadB; and the subunits for benzoyl-CoA reductase, BadDEFG. BadM is a transcriptional repressor of the *bad* operon, and BadM bound to acetamidobenzoates leads to derepression of the *bad* operon (13–15). In a *badM* mutant, the *bad* operon is constitutively expressed (14). We hypothesized that constitutive expression of the *bad* operon in EVOL-C2 and *R. palustris* RCB100 may be sufficient to enable these strains to use 3-CBA as a sole carbon source, and the ability of *R. palustris* CGA009 Δ*badM* to grow photoheterotrophically with 3-CBA as a carbon source was tested. As shown in Fig. 4A and B; Table 1, wild-type *R. palustris* CGA009 and *R. palustris* CGA009 Δ*badM* grow at similar rates when benzoate is provided as a carbon source, but only *R. palustris* CGA009 Δ*badM* can grow when 3-CBA is provided as a carbon source. This indicates that constitutive expression of the *bad* operon is sufficient for *R. palustris* to

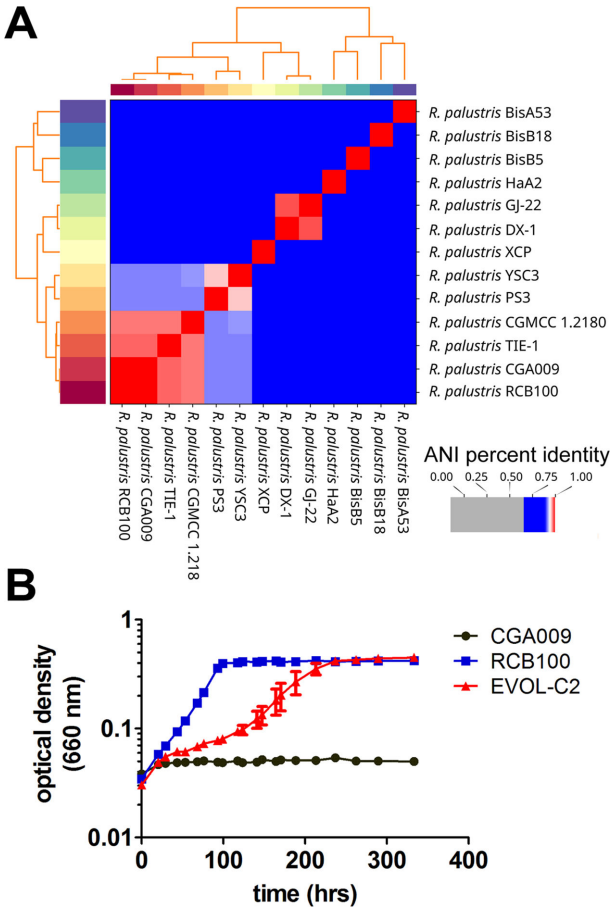


**FIG 2** Isolation of a strain of *R. palustris* CGA009 capable of growing with 3-CBA as its sole carbon source. (A) As shown by ANI, the genomes of the closely related *R. palustris* RCB100 and *R. palustris* CGA009 are >95% identical. (B) Growth of *R. palustris* CGA009, *R. palustris* RCB100, and a strain of *R. palustris* CGA009 adapted to grow with 3-CBA (EVOL-C2) in minimal medium with 1 mM 3-CBA. Data are from three independent cultures, and error bars represent standard deviation.

grow on 3-CBA. However, *R. palustris* CGA009 Δ*badM* still grew slower than RCB100 or EVOL-C2 (Table 1).

EVOL-C2 also contained a single mutation in the transcription regulator *badR*, which resulted in a protein variant with arginine 80 substituted for a leucine. BadR represses expression of the cyclohexanecarboxylate operon, which includes *aliA* (*badHI-aliAB-badK*) (16). Since a variant of AliA plays a role in 3-CBA metabolism in RCB100, we wanted to determine if inactivation of *badR* plays a role in 3-CBA metabolism. To test this, *R. palustris* CGA009 Δ*badM*, *R. palustris* CGA009 Δ*badR*, and *R. palustris* CGA009 Δ*badM badR* were grown photoheterotrophically with 3-CBA as a carbon source. We found that *R. palustris* CGA009 Δ*badM* and *R. palustris* CGA009 Δ*badM badR* grew at similar rates on 3-CBA (Fig. S2; Table S2). However, *R. palustris* CGA009 Δ*badR* was unable to grow on 3-CBA as a sole source of carbon (Fig. S2). This indicates that deletion of *badR* even when in combination with a deletion in *badM* does not enhance degradation of 3-CBA by *R. palustris* CGA009.

To determine if deletion of *badM* was sufficient to enable growth on other halogenated aromatic compounds, growth rates on 4-fluorobenzoate were also determined. Unlike 3-CBA, which requires a CoA ligase that has more activity with 3-CBA, 4-fluorobenzoate can be activated by benzoyl-CoA ligase at similar rates to benzoate, forming 4-fluorobenzoyl-CoA. 4-fluorobenzoyl-CoA is then reductively dehalogenated by benzoyl-CoA reductase to form benzoyl-CoA (Fig. 1) (8). We hypothesized that *R. palustris* CGA009

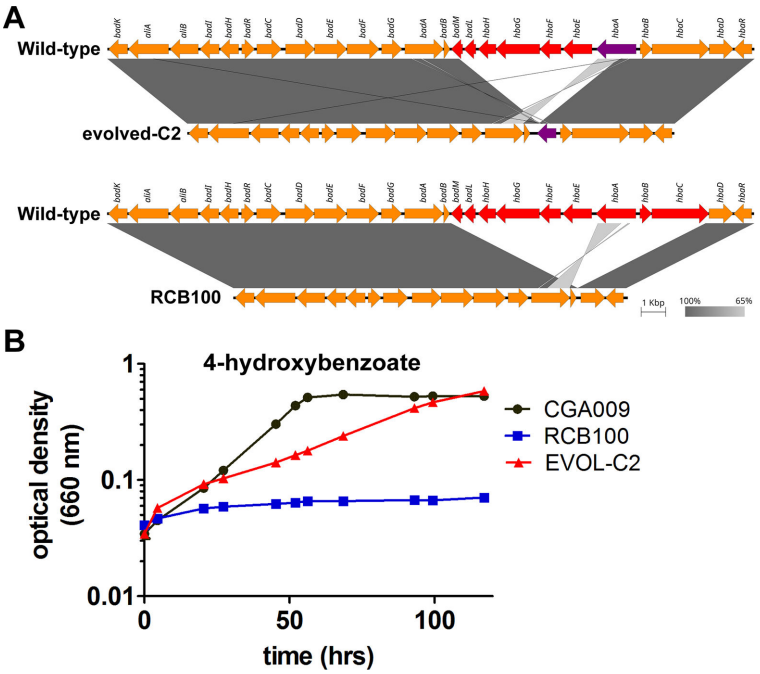


**FIG 3** *R. palustris* RCB100 and *R. palustris* EVOL-C2 have large deletions near the *bad* operon. (A) Comparison of strains CGA009 and EVOL-C2 (top) and comparison of CGA009 with RCB100 (bottom). Arrows indicate genes and their direction with the gene names listed on top of the arrows. The deleted genes are shown in red whereas partially deleted genes are shown in purple. The shaded gray gradient represents the degree of homology between the compared regions. The plots were generated with Easyfig using the BLASTn algorithm. (B) Growth of *R. palustris* CGA009, *R. palustris* RCB100, and *R. palustris* CGA009 EVOL-C2 in minimal medium with 6 mM 4-HBA. Data are from three independent cultures, and error bars represent standard deviation.

Δ*badM* and *R. palustris* RCB100 would be able to grow with 4-fluorobenzoate as a carbon source since these strains constitutively express benzoyl-CoA ligase and benzoyl-CoA reductase. As shown in Fig. 4C, *R. palustris* CGA009 is unable to use 4-fluorobenzoate as growth substrate, but *R. palustris* CGA009 Δ*badM* and RCB100 were able to grow. This indicates that deletion of *badM* enables CGA009 and RCB100 to metabolize another halogenated aromatic compound.

## Introduction of *aliA*$^{T208S}$ in *R. palustris* CGA009 Δ*badM* improves growth with 3-CBA but not 4-fluorobenzoate

Although *R. palustris* CGA009 Δ*badM* can grow when 3-CBA is provided, it grows slowly compared to RCB100 (Fig. 4B; Table 1). This is likely because, in addition to constitutive expression of benzoyl-CoA reductase, RCB100 also expresses a CoA ligase (AliA$^{T208S}$) that has 10 times more activity with 3-CBA than AliA from CGA009 (5). To test this, the *aliA*$^{T208S}$ allele was introduced into *R. palustris* CGA009 Δ*badM*. As shown in Fig. 4A and Table 1, there is no difference in growth between these strains when they are provided with benzoate as a carbon source. However, when provided with 3-CBA as a carbon source, *R. palustris* CGA009 Δ*badM aliA*$^{T208S}$ cells grew significantly faster than *R. palustris* CGA009 Δ*badM* (Fig. 4B; Table 1). Both *R. palustris* CGA009 Δ*badM* and *R. palustris* CGA009 Δ*badM aliA*$^{T208S}$ grew at similar rates on 4-fluorobenzoate, confirming that AliA$^{T208S}$ is only required for 3-CBA degradation (Fig. 4C; Table 1). This indicates that deletion of *badM* and *aliA*$^{T208S}$ is sufficient to reproduce the ability of RCB100 to degrade 3-CBA in another *R. palustris* strain.

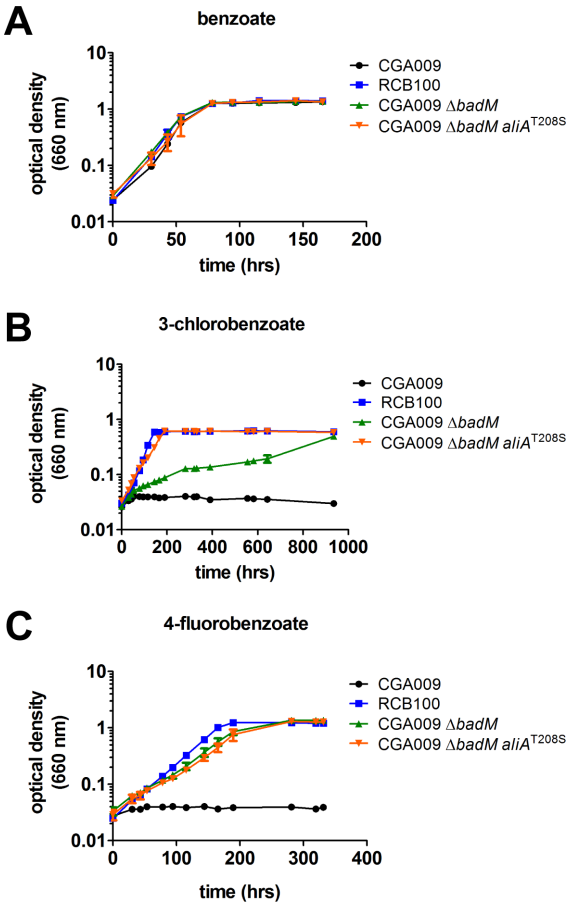


**FIG 4** Deletion of *badM* enables growth of *R. palustris* on 3-CBA and 4-fluorobenzoate, and *aliA*$^{T208S}$ enhances growth of a *badM* mutant on 3-CBA. Growth of *R. palustris* strains CGA009, RCB100, CGA009 with a deletion in *badM* (CGA009 Δ*badM*), and CGA009 Δ*badM* encoding the allele from RCB100 (CGA009 Δ*badM aliA*$^{T208S}$) in (A) minimal medium supplemented with 5.7 mM benzoate; (B) minimal medium supplemented with 3 mM 3-CBA; and (C) minimal medium supplemented with 5 mM 4-fluorobenzoate. Data are from three independent cultures, and error bars represent standard deviation.

## DISCUSSION

Using adaptive laboratory evolution, we reconstructed the evolutionary events that enabled a natural isolate to metabolize 3-CBA. We found that the environmental isolate *R. palustris* RCB100 and a laboratory strain evolved to metabolize 3-CBA, *R. palustris* EVOL-C2, contain deletions that encompass *badLM* and *hba* genes (Fig. 3). The genes in this region (*badLM-hbaAEFGH*) are encoded on the lagging strand (Fig. 3), which are sites of head-on, replication-transcription collisions. Such head-on collisions have been shown to promote the generation of duplications and deletions in bacteria, and this may explain why large deletions were found at this locus in both *R. palustris* EVOL-C2 and RCB100 (17). However, we found that deletion of a single gene in this region, *badM*, is sufficient to enable *R. palustris* to use 3-CBA as a carbon source, indicating that derepression of the *bad* genes is required for *R. palustris* to degrade 3-CBA. This is consistent with the observation that *R. palustris* strains can co-metabolize 3-CBA and benzoate despite being unable to metabolize 3-CBA as the sole source of carbon (3, 9, 10).

The fact that *R. palustris* CGA009 Δ*badM* can grow on 3-CBA implies that native CoA ligases have sufficient activity with 3-CBA to enable 3-CBA metabolism. This is surprising because the ability to degrade 3-CBA is thought to be restricted to bacterial strains encoding a CoA ligase with increased affinity for 3-CBA (6, 18). *R. palustris* CGA009 encodes three CoA ligases involved in anaerobic aromatic compound degradation:

**TABLE 1** Growth rates of *R. palustris* strains on aromatic compounds used in this study

| Genotype | Doubling time (h)[a] | | | |
|---|---|---|---|---|
| | Benz | 3-CBA | 4-FlB | 4-HBA |
| RCB100 | 10 (0.3) | 35 (1.7) | 33 (0.9) | NG[b] |
| CGA009 | 10 (0.2) | NG | NG | 14 (0.4) |
| CGA009 EVOL-C2 | 9 (1.3) | 89 (5.1) | ND[c] | 35 (0.6) |
| CGA009 Δ*badM* | 11 (0.6) | 178 (6.8) | 43 (2.6) | ND |
| CGA009 Δ*badM aliA*$^{T208S}$ | 12 (2.2) | 39 (3.1) | 46 (0.9) | ND |

[a]All doubling times are the averages from three independent experiments and are shown in hours with standard deviation in parentheses. Benz, 5.7 mM benzoate; 3-CBA, 3 mM 3-chlorobenzoate; 4-FlB, 5 mM 4-fluorobenzoate; and 4-HBA, 6 mM 4-hydroxybenzoate.
[b]NG, no growth observed.
[c]ND, not determined.

benzoyl-CoA ligase, BadA; 4-hydroxybenzoyl-CoA ligase, HbaA; and cyclohexanecarboxylate-CoA ligase, AliA. HbaA is likely not involved since *hbaA* is deleted in *R. palustris* EVOL-C2 and *R. palustris* RCB100. However, both *badA* and *aliA* are intact in *R. palustris* EVOL-C2, and BadA and AliA have been shown to have some activity against 3-CBA *in vitro* (5, 19). BadA and AliA are also expressed in a *badM* mutant grown with 3-CBA as a carbon source. Deletion of *badM* leads to constitutive expression of BadA, and BadA activity is even higher in a *badM* mutant compared to induced wild-type cells (14, 20). AliA activity has also been observed in a *badA* mutant grown with benzoate, indicating that both AliA and BadA are present in cells carrying out benzoyl-CoA degradation (21). This suggests that there is enough promiscuous activity by BadA and AliA in a *badM* mutant to enable growth on 3-CBA, and a CoA ligase specific to 3-CBA is not a requirement *per se*.

Together, these data point toward a model wherein *R. palustris* RCB100 first acquired a deletion that inactivated *badM*, leading to constitutive expression of BadA, AliA, and benzoyl-CoA reductase. While the promiscuous activity of the CoA ligases is adequate for enabling 3-CBA degradation, CoA activation still represents a potential rate-limiting step, creating selective pressure to acquire a mutation that enhances CoA ligase activity with 3-CBA. This aligns with the presence of AliA$^{T208S}$, a CoA ligase that has higher activity with 3-CBA, in *R. palustris* RCB100. Introduction of the *aliA* allele from *R. palustris* RCB100 into *R. palustris* CGA009 Δ*badM* significantly increased growth on 3-CBA, resulting in a growth rate similar to *R. palustris* RCB100. This establishes these two mutations as sufficient to reconstruct the genetic requirements in *R. palustris* RCB100 for 3-CBA degradation.

Our findings also indicate that *R. palustris* is unable to sense halogenated aromatic compounds since deletion of *badM* is required for *R. palustris* to grow on these compounds. Repression of BadM is relieved when it is bound to acetamidobenzoates, which are produced by the acetylation of aminobenzoates by BadL (13). When grown with 3-CBA or 4-fluorobenzoate, accumulation of acetaminobenzoates must not occur in *R. palustris*, and BadM repression is not relieved. It is difficult to speculate why BadM repression is not relieved in the presence of halogenated compounds as it is still unclear why acetaminobenzoates accumulate when cells are grown with benzoate. The growth medium for *R. palustris* contains 4-aminobenzoate (11 mM) for folate biosynthesis, and 4-aminobenzoate is a substrate for BadL (13, 22). Why derepression of BadM only occurs in the presence of aromatic compounds like benzoate but not in the presence of halogenated aromatic compounds in a medium containing 4-aminobenzoate is unclear.

This is the first evolutionary reconstruction of an environmental isolate capable of using halogenated aromatic compounds. Adaptive laboratory evolution has provided numerous examples of how regulatory mutations or variants of a promiscuous enzyme can enable degradation of man-made compounds (23–31). Here, we show that both bottlenecks, gene regulation and substrate specificity of a promiscuous enzyme, were overcome to enable efficient degradation of 3-CBA.

## MATERIAL AND METHODS

### Bacterial strains and culture conditions

All bacterial strains used in the current study are listed in Table 2. *R. palustris* strains were grown under anoxic conditions in 10 mL of minimal mineral photosynthetic medium (PM) in sealed Hungate tubes (22, 32). Carbon substrates from anoxic, sterile stock solutions were added after autoclaving. Carbon sources were added to the following final concentrations where indicated: 10 mM succinate, 20 mM acetate, 5.7 mM benzoate, 1 to 3 mM 3-chlorobenzoate, 5 mM 4-fluorobenzoate, and 6 mM 4-HBA. All liquid cultures were grown at 30°C and provided light (30 µmol photons/$m^2$/s) from a 60 W halogen light bulb (General Electric). Cultures were initially grown in PM with succinate and then diluted in PM with the stated carbon source for growth analysis. Optical density was determined at 660 nm using a Genesys 50 UV-visible spectrophotometer. *Escherichia coli* strains were grown in lysogeny broth medium at 37°C. When necessary, *R. palustris* and *E. coli* cultures were supplemented with gentamicin (Gm) at concentrations of 100 and 20 µg/mL, respectively.

### Experimental evolution *of R. palustris* CGA009 and isolation of evolved *R. palustris* EVOL-C2

A single colony of *R. palustris* CGA009 was picked up with a sterilized toothpick and resuspended in 200 µL of PM medium in a sterile Eppendorf tube before the suspended cells were introduced into sealed Hungate tubes containing 10 mL of PM supplemented with acetate. To adapt *R. palustris* CGA009 to grow on 3-CBA, cells were inoculated into four tubes containing PM supplemented with 5.7 mM benzoate and 1 mM 3-CBA as described in reference (11). Cells were grown for 3 weeks under light, before they were transferred to PM medium supplemented with 2.85 mM benzoate and 1.5 mM 3-CBA. Cells were grown in the light for 5 weeks and then transferred into PM medium supplemented with 1.5 mM benzoate and 2.5 mM 3-CBA. Cells were grown in the light for 5 weeks before they were transferred to PM medium with 0.5 mM benzoate and 3.5 mM 3-CBA. Cells were grown in the light for 5 weeks and transferred into PM supplemented with 3.5 mM 3-CBA. After 9 weeks, a single replicate grew, and 100 µL of cells were spread on plates containing PM with 5.7 mM benzoate. After 3 weeks of incubation, 13 random colonies were selected and grown in Hungate tubes with PM supplemented with 1 mM 3-CBA. EVOL-C2 was one of the 13 colonies that consistently grew in PM supplemented with 1 mM 3-CBA. After repeated confirmation of growth on liquid PM supplemented with 1 mM 3-CBA, 100 µL of EVOL-C2 was transferred to PM liquid medium supplemented with 20 mM acetate and 0.1% yeast extract. Once fully grown, a glycerol stock was prepared and stored at −80°C, and genomic DNA was extracted for sequencing as described below.

### Genomic DNA extraction and whole genome sequence analyses

Genomic DNA was extracted as described previously (33). Briefly, *R. palustris* EVOL-C2 was grown in 10 mL PM medium with 20 mL acetate. Freshly grown *R. palustris* cells were harvested with centrifugation at 12,000 rpm for 10 minutes, and genomic DNA was extracted from cell pellets as described (6, 8). Both Illumina and Nanopore libraries were prepared at Microbial Genome Sequencing Center (MiGS; https://www.migscenter.com/), using the workflows reported previously (6, 9), before sequencing on NextSeq 550 and MinION v.9.4.1 flow cell, respectively. Base calling was performed using guppy v.4.2.2 with default parameters + *effbaf8*. The quality control and adapter trimming for Illumina reads were performed with bcl2fastq v.2.20.0.445 (10), whereas porechop v.0.2.3_seqan2.1.1 (11) was used for Oxford Nanopore Technology (ONT) sequencing reads. The Illumina data set comprised 5,063,564 reads pairs and 1,632,037,238 bases whereas Nanopore sequencing produced 116,959 reads and 303,624,697 bases.

A hybrid genome assembly was generated from Illumina and ONT reads with Unicycler v.0.4.8 (12), and QUAST v.5.0.2 (13) was used to obtain genome assembly

**TABLE 2** Strains, plasmids, and primers used in this study

| Strain, plasmid, or primer | Genotype, description, or primer sequence (5′ to 3′) | Reference, origin, or description |
|---|---|---|
| *R. palustris* strains | | |
| CGA009 | Wild-type strain; spontaneous $Cm^R$ derivative of CGA001 | (32) |
| RCB100 | Isolated on 3-CBA near Cascadilla Creek, Ithaca, NY | (3) |
| EVOL-C2 | CGA009 adapted to grow on 3-CBA; genotype in Table S1 | This study |
| CGA131 | CGA009 Δ*badM*; CGA009 containing an in-frame deletion of *badM* (*rpa0663*) | (14) |
| CGA609a | CGA009 Δ*badR*; CGA009 containing an in-frame deletion of *badR* (*rpa0655*) | (16) |
| CGA139 | CGA009 Δ*badM badR*; CGA009 containing an in-frame deletion of *badR* and *badM* | Gift from Caroline Harwood |
| $aliA^{T208S}$ | CGA009 encoding the *aliA* allele from RCB100 | This study |
| Δ*badM* $aliA^{T208S}$ | CGA131 encoding the *aliA* allele from RCB100 | This study |
| *E. coli* strains | | |
| S17-1 | *thi pro hdsR hdsM*$^+$ *recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7) | (3) |
| Plasmids | | |
| pJQ200SK | $Gm^R$, *sacB*; mobilizable suicide vector | (4) |
| pJQ-$aliA^{T208S}$ | $Gm^R$; used for allelic exchange of the *aliA* allele from *R. palustris* RCB100 | This study |
| Primers | | |
| pJQ200SK-*aliA*F | GCAGCAGAACGGCGTCGAACTCCAT-CTCCAGCTTTTGTTCCCTTTAGTGAGGG | This study |
| pJQ200SK-*badK*R | GATGTGCTCAACGCGCTCAACGATG-GCTCTAGAACTAGTGGATCCCCCGG | This study |
| *aliA*F | TCACTAAAGGGAACAAAAGCTGGAG-ATGGAGTTCGACGCCGTTCTGC | This study |
| *badK*R | CCGGGGGATCCACTAGTTCTAGAGC-CATCGTTGAGCGCGTTGAGCACATC | This study |

statistics. Genome assembly resulted in two circular contigs of 5,452,700 bp and 8,427 bp that represented chromosome and plasmid, respectively. The GC (%) nucleotide content of the assembled genome was 65.03, whereas the length of the genome was 5,461,127 bp. The assembly annotation was performed with NCBI Prokaryotic Genome Annotation Pipeline. To predict mutations in strains EVOL-C2 and RCB100 in comparison to strain CGA009, we used breseq v.0.36.0 (34).

We used PYANI v.0.2.10 [(35); https://github.com/widdowquinn/pyani] to calculate average nucleotide identity among 13 selected *R. palustris* genomes based on BLASTN+ (ANIb) method. Easyfig v.2.2.2 (36) was used to generate genome comparison visualization plots.

## Genetic manipulation of *R. palustris*

A construct containing the *aliA* locus from *R. palustris* RCB100 was constructed for allelic exchange using the primers shown in Table 2. Genomic DNA purified from *R. palustris* RCB100 was used as a template for PCR amplification using Phusion High-Fidelity DNA polymerase (New England Biolabs) with primers *aliA*F and *badK*R (Table 2). Using homologous recombination as described in reference (37), the resulting product was incorporated into linearized pJQ200SK generated from PCR amplification with primers pJQ200SK-*aliA*F and pJQ200SK-*badK*R (Table 2). The resulting plasmid was mobilized into *R. palustris* CGA009 by conjugation with *E. coli* S17-1, and double-crossover events for allelic exchange were accomplished using a previously described selection and screening strategy (38). Replacement of *aliA* in *R. palustris* CGA009 with the *aliA* locus from *R. palustris* RCB100 was verified using PCR followed by Sanger sequencing (Azenta Life Sciences, Burlington, MA).

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## AUTHOR CONTRIBUTIONS

Irshad Ul Haq, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review and editing | Annika Christensen, Investigation, Validation | Kathryn R. Fixen, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review and editing

## DATA AVAILABILITY

The *R. palustris* RCB100 genome sequence is available at NCBI under the accession number GCF_016584445.1. The *R. palustris* CGA009 genome sequence is available under the accession number GCF_000195775.2, and the *R. palustris* CGA009 EVOL-C2 genome sequence is available under the accession number GCA_031600295.1. Raw sequencing data for EVOL-C2 is available at NCBI under the accession numbers SRR17562209 (Oxford Nanopore) and SRR17562208 (Illumina). The accession number for the EVOL-C2 sequencing BioProject is PRJNA796295, and the Biosample accession number is SAMN24839554.

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Fig. S1 and S2, Tables S1 and S2 (AEM02104-23-s0001.docx).** Supplemental material.

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