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Genetic Analysis of Trimethylamine *N*-Oxide Reductases in the Light Organ Symbiont *Vibrio fischeri* ES114[▽]

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Trimethylamine *N*-oxide (TMAO) reductases are widespread in bacteria and often function in anaerobic respiration. The regulation and expression of TMAO reductase operons have been well studied in model genera such as *Escherichia*, *Shewanella*, and *Rhodobacter*, although TMAO reductases are present in many other bacteria, including the marine *Vibrio* species. The genome sequence of *Vibrio fischeri* revealed three putative TMAO reductase operons, and a previous report identified TMAO reductase activity in symbiotic *V. fischeri* isolates associated with the light organs of adult Hawaiian bobtail squid, *Euprymna scolopes*. We examined the roles and regulation of these three operons using mutational analyses and promoter-reporter fusions. We found that the *torECA* promoter, and to a lesser extent the *torYZ* and *dmsABC* promoters, were active during symbiotic colonization of juvenile *E. scolopes*; however, a *V. fischeri* strain lacking TMAO reductase activity displays no discernible colonization defect over the first 48 h. Our studies also revealed that *torECA* has the most active promoter of the putative TMAO reductase operons, and TorECA is the major contributor to TMAO-dependent growth in *V. fischeri* under the conditions tested. Interestingly, the transcriptional regulation of TMAO reductase operons in *V. fischeri* appears to differ from that in previously studied organisms, such as *Escherichia coli*, which may reflect differences in gene arrangement and bacterial habitat. This study lays the foundation for using *V. fischeri* as a model system for studying TMAO reductases in the *Vibrionaceae*.

Trimethylamine *N*-oxide (TMAO) can be used by various bacteria as a terminal electron acceptor during anaerobic respiration. TMAO is commonly found in marine environments, including in the tissues of marine animals, where it is used as an osmolyte (5) and can reach levels of mmol kg^{-1} of wet weight (21, 41). Much of our understanding of the regulation and function of TMAO reductases comes from studies of bacteria from the genera *Escherichia*, *Shewanella*, and *Rhodobacter* (24), although TMAO reductases can also be found in other genera. A previous report demonstrated that marine *Vibrio* species are capable of reducing TMAO (27), and genome sequencing projects are revealing that TMAO reductases are common within the *Vibrionaceae*, which includes many economically and/or medically important species. However, the regulation of these operons and their relative contribution to TMAO reduction in *Vibrio* species have not been studied, despite the fact that TMAO is most common in marine environments (5). In addition, many of the previous studies have focused on TMAO reduction during growth in laboratory cultures, leaving the relevance of TMAO reduction in a natural (e.g., marine host associated) environment largely unexplored. To address these areas, we have used *Vibrio fischeri* as a model organism to examine TMAO reduction in the *Vibrionaceae*. Because the symbiosis between *V. fischeri* and its symbiotic partner *Euprymna scolopes* (40) can be reconstituted in the lab (29), we were able to address how TMAO reduction contributes to bacterial growth in a natural habitat where TMAO is presumably present.

The genome sequence of *V. fischeri* (31) revealed that this organism likely has three TMAO reductase operons, and previous work by others identified TMAO reductase activity in *V. fischeri* cells grown under laboratory conditions and during symbiotic growth in association with *E. scolopes* light organ tissue (27). These data suggest that TMAO reduction may be an important respiratory pathway in *V. fischeri* during growth in environments where TMAO is present. Our goals were to characterize the regulation of each of the three putative TMAO reductase operons in response to growth conditions, to determine whether known regulatory proteins influenced the promoter activity of each operon, and to determine the contribution of each putative reductase to TMAO-dependent growth during the culture of *V. fischeri* under both batch-culture and symbiotic conditions. This study represents the first in-depth characterization of TMAO-based respiration in a marine organism other than *Shewanella* spp.

MATERIALS AND METHODS

Reagents. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA), and chemicals were obtained from Sigma-Aldrich (St. Louis, MO). PCR analysis was performed using KOD HiFi DNA polymerase (Novagen, San Diego, CA), using the manufacturer's suggested reaction conditions. All PCR-amplified DNA fragments were verified by sequencing at the University of Michigan DNA Sequencing Core Facility or at the Oklahoma Medical Research Foundation DNA Sequencing Center. Primer sequences are available upon request.

Strains and plasmids. Strains and plasmids used in this study are given in Table 1. General cloning procedures were used, and plasmids were transformed and maintained in *E. coli* strains DH5α (17) or DH5α λ*pir* (13). Constructs containing the RP4 origin of transfer were introduced into *V. fischeri* isolates using triparental mating as previously described (37).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
Vibrio fischeri		
ES114	Wild-type isolate from *E. scolopes* light organ tissue	7
AKD800	ES114 Δ*torECA* (allele exchanged from pAKD800)	This study
AKD801	ES114 Δ*torYZ* (allele exchanged from pAKD801)	This study
AKD802	ES114 Δ*dmsABC* (allele exchanged from pAKD802)	This study
AKD803	ES114 Δ*torECA* Δ*torYZ*	This study
AKD804	ES114 Δ*torECA* Δ*dmsABC*	This study
AKD805	ES114 Δ*torYZ* Δ*dmsABC*	This study
AKD806	ES114 Δ*torECA* Δ*torYZ* Δ*dmsABC*	This study
AKD807	ES114 Tn*7* P_{torYZ}-*lacZ* insertion	This study
AKD808	ES114 Tn*7* P_{torECA}-*lacZ* insertion	This study
AKD809	ES114 Tn*7* P_{dmsABC}-*lacZ* insertion	This study
JB1	ES114 Δ*fnr*	J. L. Bose
KV1585	ES114 *torR*::pKV174	18
Escherichia coli		
DH5α	F′/*endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80d*lacI*Δ(*lacZ*)M15]	17
DH5α λ*pir*	λ*pir* derivative of DH5α	13
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir*	33
Plasmids		
pVSV105	*E. coli-V. fischeri* shuttle vector, RP4 *oriT*, pES213 and R6K replication origins, Cm^r	14
pVSV209	*E. coli-V. fischeri* shuttle vector, RP4 *oriT*, pES213 and R6K replication origins, promoterless *gfp* and Cm^r, constitutive *rfp*, Kn^r	14
pVSV209torECA	pVSV209 with the *torECA* promoter region	This study
pVSV209torYZ	pVSV209 with the *torYZ* promoter region	This study
pVSV209dmsABC	pVSV209 with the *dmsABC* promoter region	This study
pAKD701	*gfp* and Cm^r-containing StuI-*San*DI fragment in pVSV209 was replaced with a StuI-SanDI *lacZ* fragment PCR-amplified from *E. coli* MG1655 using lacZF and lacZR, *rfp* removed by NotI digestion followed by self-ligation	This study
pAKD701torECA	pAKD701 with the *torECA* promoter region	This study
pAKD701torYZ	pAKD701 with the *torYZ* promoter region	This study
pAKD701dmsABC	pAKD701 with the *dmsABC* promoter region	This study
pAKD800	Δ*torECA* allele, R6K and ColE1 replication origins, RP4 *oriT*, Em^r, Kn^r	This study
pAKD801	Δ*torYZ* allele, R6K and ColE1 replication origins, RP4 *oriT*, Em^r, Kn^r	This study
pAKD802	Δ*dmsABC* allele, R6K and ColE1 replication origins, RP4 *oriT*, Em^r, Kn^r	This study
pAKD803	pVSV105 containing the *torECA* complementing fragment	This study
pEVS104	Conjugative helper plasmid, R6K replication origin, Kn^r	37
pEVS107	pEVS94S derivative, mini-Tn*7*, mob, Em^r, Kn^r	23
pEVS107torYZ	pEVS107 containing P_{torYZ}-*lacZ* PCR amplified from pAKD701torYZ	This study
pEVS107torECA	pEVS107 containing P_{torECA}-*lacZ* PCR amplified from pAKD701torECA	This study
pEVS107dmsABC	pEVS107 containing P_{dmsABC}-*lacZ* PCR amplified from pAKD701dmsABC	This study

[a] Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance; Em^r, erythromycin resistance; *rfp*, red fluorescent protein gene.

***V. fischeri* strains lacking TMAO reductase operons.** Strains lacking one, two, or all three of the putative operons were constructed by allelic exchange (10), resulting in in-frame deletion mutants. Briefly, approximately 1.6 kb of DNA upstream of the start codon for each operon was PCR amplified and fused to an approximately 1.6-kb DNA fragment downstream of the stop codon for each operon using an engineered restriction site, resulting in replacement of the operon with a 6-bp restriction enzyme recognition site between the start and stop codons.

Green fluorescent protein reporter plasmids. Approximately 500 bp (*torECA*) or 300 bp (*torYZ* and *dmsABC*) of DNA upstream of the ATG start codon for the first gene in each operon was PCR amplified using primers engineered to contain recognition sites for SalI and AvrII restriction endonucleases. These fragment sizes were chosen to reflect the intergenic region between the operon of interest and the nearest upstream coding sequence. These fragments were cloned into the SalI and AvrII restriction sites of pVSV209 (14), generating transcriptional promoter fusions to a promoterless *gfp*. These plasmids contain the *V. fischeri* pES213 origin of replication and are present in approximately 10 copies per chromosome in *V. fischeri* isolates (13).

***lacZ* reporter plasmids.** The promoter-containing fragments described in the previous section were similarly PCR amplified and cloned into pAKD701 to generate transcriptional promoter fusions to *lacZ*. pAKD701 was constructed by replacing the *gfp* and chloramphenicol resistance gene-containing StuI-SanDI fragment in pVSV209 (14) with a StuI-SanDI *lacZ* fragment PCR amplified from *E. coli* MG1655 and removal of an *rfp*-containing NotI fragment. These plasmids contain the *V. fischeri* pES213 origin of replication and are present in approximately 10 copies per chromosome in *V. fischeri* isolates (13).

Chromosomal promoter-*lacZ* fusion reporters. The TMAO reductase promoter regions fused to the *lacZ* reporter on pAKD701torECA, pAKD701torYZ, or pAKD701dmsABC were PCR amplified and cloned into pEVS107 (23), generating mini-Tn*7* constructs containing the respective TMAO reductase promoters fused to *lacZ*. The chromosomal insertion was performed as described previously (23).

Growth media. *V. fischeri* strains were grown at 24 or 28°C in LBS (35) or mineral salts medium (MS), which contained (per liter) 1.0 g Casamino Acids, 6 ml 50% glycerol, 378 μl 1 M $NaPO_4$ (pH 7.5), 50 ml 1 M Tris (pH 8.0), 6 mg $FeSO_4$-$7H_2O$, 13.6 g $MgSO_4$-$7H_2O$, 0.83 g KCl, 19.5 g NaCl, 1.62 g $CaCl_2$-$2H_2O$. When noted, 20 mM glucose was added to MS. For overnight cultures harboring plasmids, 100 μg ml^{-1} of kanamycin was added to the growth medium. For anaerobic growth, 20 ml of medium was placed in sealed 165-ml bottles, and the bottles were sparged with a 5% H_2, 10% CO_2, and an 85% N_2 gas mixture prior to autoclaving, except where noted.

Visualization of promoter-*gfp* reporter activity in juvenile squid light organs.

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TABLE 2. Activity of TMAO reductase promoter-*lacZ* fusions

Conditions[a]	Promoter activity in Miller units[b]					
	P_{torECA}-*lacZ*		P_{torYZ}-*lacZ*		P_{dmsABC}-*lacZ*	
	Without TMAO	With TMAO	Without TMAO	With TMAO	Without TMAO	With TMAO
Aerobic						
LBS						
Mid-log phase	120 (±6)[d]	360 (±35)	8 (±1)	7 (±1)	2 (±0.1)[c]	5 (±1)
Stationary phase	170 (±6.3)[c]	390 (±58)	100 (±14)[c]	60 (±4)	3 (±0.3)[c]	5 (±0.4)
MS with glucose						
Mid-log phase	170 (±37)	220 (±39)	6 (±1)	5 (±1)	2 (±0.1)	2 (±0.4)
Stationary phase	340 (±16)	481 (±145)	20 (±5)	10 (±1)	4 (±1)[c]	2 (±0.2)
MS without glucose						
Mid-log phase	300 (±33)	440 (±73)	17 (±1.1)[c]	12 (±1.2)	3 (±0.1)	2 (±0.2)
Stationary phase	810 (±63)	890 (±65)	30 (±1)[d]	17 (±0.9)	5 (±0.3)[d]	3 (±0.2)
Anaerobic						
LBS						
Mid-log phase	340 (±1)	500 (±86)	90 (±1)[d]	60 (±6)	70 (±6)[d]	10 (±1)
Stationary phase	320 (±15)[d]	1,220 (±81.9)	100 (±3.5)[d]	200 (±13)	70 (±2)[d]	40 (±2)
MS with glucose						
Mid-log phase	30 (±5)[d]	160 (±23)	70 (±9)[d]	10 (±2)	76 (±21)[c]	6 (±1)
Stationary phase	50 (±3)	604 (±234)	110 (±7.2)[d]	70 (±3)	170 (±11)[d]	30 (±2)
MS without glucose						
Mid-log phase	ND	780 (±29)	ND	120 (±22)	ND	80 (±5)
Stationary phase	ND	3,170 (±164)	ND	280 (±12)	ND	100 (±9)

[a] Culture conditions included growth in LBS (rich medium) or MS, either aerobically or anaerobically, and with or without 40 mM TMAO. OD_{595} measurements were used to determine the mid-log and stationary phases (see Table 3).

[b] Promoter activity is reported for the average of three independent cultures (± standard error of the mean). Significant differences between Miller unit values for a particular promoter with and without TMAO under specific growth conditions were determined using Student's *t* test. ND, not determined; *V. fischeri* will not grow under these conditions.

[c] $P < 0.05$.

[d] $P < 0.01$.

Aposymbiotic *E. scolopes* hatchlings were inoculated with *V. fischeri* ES114 containing pVSV209, pVSV209torECA, pVSV209torYZ, or pVSV209dmsABC, as described previously (14). At the indicated time points, individual animals were dissected and visualized using a Nikon (Melville, NY) Eclipse E600 epifluorescence microscope outfitted with a Nikon Chroma 51004 v2 fluorescein isothiocyanate/tetramethyl rhodamine isothiocyanate filter cube, a Nikon Chroma 41017 Endow green fluorescent protein filter cube, and a Nikon Coolpix 5000 camera. All images were treated equally, with cropping and brightness reduction performed in Microsoft PowerPoint 2003.

Assays for symbiotic competence. Aposymbiotic *E. scolopes* hatchlings were inoculated with an approximately 1:1 mix of AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) and its parent, ES114, as previously described (14). After 48 h, individual animals were homogenized and dilution plated. Plates were incubated aerobically at 28°C overnight, and individual colonies were patched to plates containing TMAO and incubated anaerobically in GasPak jars (BD, Franklin Lakes, NJ) at 28°C for 16 to 20 h to determine the ratio of AKD806 (*V. fischeri* patches that did not grow) to ES114. The ability of AKD806 to grow after patching was confirmed in a subset of the samples by incubating a replicate plate aerobically. The ability of AKD806 to compete with ES114 is presented as a relative competitiveness index (RCI), which is the ratio of AKD806 to ES114 in the host divided by the ratio of these strains in the inoculum. An RCI not significantly different from 1 indicates the strains compete equally well during host colonization. Three independent experiments were performed, and the data were combined. Statistical significance was determined using Student's *t* test on log-transformed data.

Anaerobic growth curves. *V. fischeri* strains ES114, AKD803 (Δ*torECA* Δ*torYZ*), AKD804 (Δ*torECA* Δ*dmsABC*), AKD805 (Δ*torYZ* Δ*dmsABC*), and AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) were grown at 24°C with either 4 or 40 mM TMAO in MS, in MS supplemented with glucose, or in LBS. Anaerobic bottles were inoculated with a 1,000-fold dilution of an aerobically grown overnight culture of the respective strain, and the optical density was measured at 595 nm. Each experiment consisted of three independent cultures for each strain and was repeated three times. In each case, one representative experiment is shown.

β-Galactosidase assays testing the influence of environmental conditions on promoter-*lacZ* transcriptional fusions. Assays were performed as described previously (10), using a modified Miller assay. Cells were grown aerobically or anaerobically in LBS, MS, or MS supplemented with glucose, either with or without 40 mM TMAO. For aerobic growth, an overnight culture (16 h) was diluted 1,000-fold in 15 ml of medium in a 125-ml Erlenmeyer flask. For anaerobic growth, an aerobic overnight culture (16 h) was diluted 1,000-fold into 20 ml of medium in sealed anaerobic 165-ml bottles. Samples were collected at the mid-log and stationary phases, with the specific optical density at 595 nm varying per medium type and growth conditions. Each experiment consisted of three independent cultures for each strain and was repeated three times. One representative experiment of the three is shown. Average Miller unit values for the negative control (*V. fischeri* harboring pAKD701) for each growth condition were subtracted prior to calculating the average and the standard error. Values for the negative control varied per growth condition and ranged from 0.2 to 1.4 Miller units.

β-Galactosidase assays testing the influence of known regulatory proteins on promoter-*lacZ* transcriptional fusions. Assays were performed as described in the previous section. Culture tubes (18-mm in diameter) containing 3 ml of LBS were inoculated with 3 μl of an overnight culture and placed in sealed canisters with a GasPak EZ anaerobe pouch system (BD, Franklin Lakes, NJ) to achieve anaerobic growth conditions. The canisters were placed at 24°C until the cultures reached the stationary phase. For experiments with KV1585, 40 mM of TMAO was added to the LBS when indicated. Cultures grown in the GasPak system displayed levels of promoter activity similar to those grown in anaerobic bottles (e.g., Table 2 and data not shown); therefore, the GasPak system was used for these assays. Each experiment consisted of three independent cultures for each strain, and one representative experiment of three is shown. The average Miller unit values are presented as described in the previous section.

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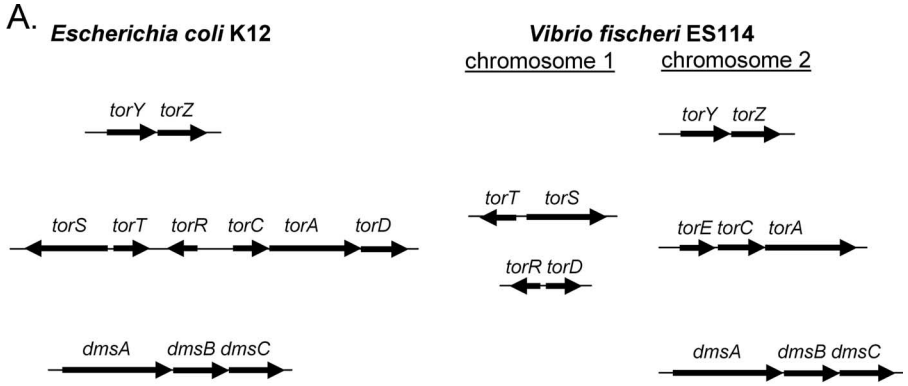

B.

	torYZ	torECA	torTS	torRD	dmsABC
Vibrio fischeri ES114	chromosome 2 VFA0189-88	chromosome 2 VFA0297-99	chromosome 1 VF1619-20	chromosome 1 VF1570-1	chromosome 2 VFA0082-80
V. cholerae El Tor N16961	chromosome 1 VC1950-1	chromosome 1 VC1692-4	chromosome 2 VCA0709-10	chromosome 1 VC1719-20	absent
V. vulnificus YJ016	chromosome 1 VV1374-5	absent	chromosome 2 VVA0632-3	chromosome 1 VV2303-4	absent
V. parahaemolyticus RIMD 2210633	chromosome 2 VPA0127-8	chromosome 1 VP1160-2	chromosome 2 VPA0674	chromosome 1 VP1031-2	chromosome 1 VP1447-9

FIG. 1. TMAO reductase operon structure in *E. coli* and *Vibrio* species. (A) Comparison of gene organization in *E. coli* and *V. fischeri*. (B) Presence and location of TMAO reductase operons in representative *Vibrio* species. Chromosomal location and ORF numbers are indicated for individual genes.

RESULTS

The annotation of the *V. fischeri* ES114 genome (31) identified three putative TMAO reductase operons composed of open reading frames (ORFs) VFA0297-VFA0299, VFA0189-VFA0188, and VFA0082-VFA0080. We further used BLAST (1) to identify sequence similarity to known TMAO reductase operons in *E. coli* and sequence similarity to TorE in "*Shewanella massilia*" and found homologs in *V. fischeri* ranging from 37 to 76% amino acid identity over >93% of the respective protein. Based on this information, we assigned these ORFs in *V. fischeri* as TorECA, TorYZ, and DmsABC, respectively.

Based on these analyses, ES114 appears to contain a suite of TMAO reductase-like operons similar to those found in *E. coli* (Fig. 1A). However, for the operon encoding TorA, in *E. coli* the regulatory elements (TorS, TorT, and TorR) and the structural components (TorC, TorA, and TorD) are encoded contiguously on the genome, whereas in *V. fischeri*, the genes are arranged in three separate operons, one of which is on a separate chromosome (Fig. 1A). In addition, unlike in *E. coli*, in *V. fischeri* the TorA operon includes *torE*, a gene also found in *Shewanella* species (12). The function of TorE is currently unknown, but it is predicted to be a small membrane protein (12). Interestingly, the presence of the TMAO reductases varies between *Vibrio* species, with *Vibrio vulnificus* lacking *torECA* and both *V. vulnificus* and *Vibrio cholerae* lacking *dmsABC* (Fig. 1B). When the operons are present, their chromosomal location is not consistent among all species (Fig. 1B). In addition, one alternative operon arrangement has been identified in the unfinished genomes of *Photobacterium profundum* 3TCK and "*Vibrio angustum*" S14, where TorR and TorD are encoded contiguously with TorYZ on the chromosome. Although the location and presence of TMAO reductase operons vary between the *Vibrio* species shown in Fig. 1, *V. fischeri* does appear to encode the full set of known TMAO reductases and regulatory elements, making it a good starting point for understanding the regulation and use of the TMAO reductases in *Vibrio* species and how these differ from the currently characterized systems in *E. coli* and other organisms.

Testing the expression and role of TMAO reductase operons during symbiotic colonization. To gain insight into the function of each TMAO reductase in *V. fischeri* and the importance of TMAO reduction during growth in a natural environment (during symbiotic colonization), we constructed plasmid-based promoter-*gfp* reporters and in-frame deletion mutants.

To determine whether TMAO reductase operon promoters were active during host colonization, we inoculated aposymbiotic juvenile squid with ES114 carrying the pVSV209-based (14) *gfp* transcriptional reporter plasmids and qualitatively monitored colonization and promoter activity using epifluorescence microscopy. Seventy-two hours after inoculation, the juvenile light organs were fully colonized by *V. fischeri* (Fig. 2D to G), but promoter activity varied among the operons (Fig. 2H to K). The *torECA* promoter appeared to be most active, with relatively slight activity for P_{torYZ} and P_{dmsABC}. Similar levels of promoter activity were observed at 24 and 48 h postinoculation (data not shown), suggesting that relative promoter activity does not change during the early stages of colonization.

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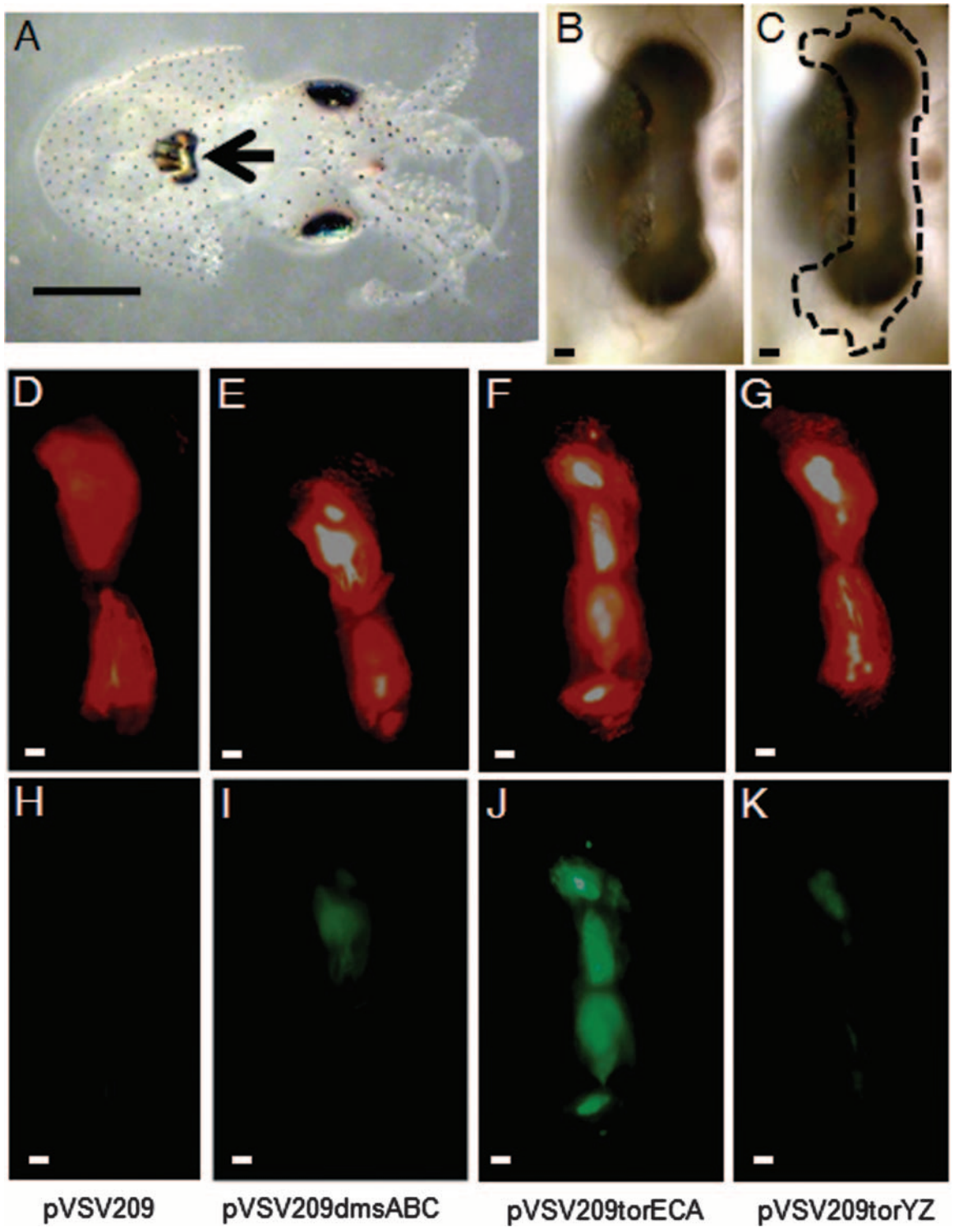

FIG. 2. Expression of TMAO reductase operon promoter-*gfp* reporters during growth in the host light organ. (A) Light microscopy image of the ventral side of a juvenile squid. The arrow points to the location of the light organ tissue. The black bar represents 1 mm. (B) Higher magnification of the light organ tissue area depicted in panel A. The black bar represents approximately 10 μm. (C) Same image as panel B but with the location of the transparent light organ tissue highlighted. Epifluorescence microscopy was used to image juvenile squid light organs infected with *V. fischeri* containing pVSV209 (D, H), pVSV209dmsABC (E, I), pVSV209torECA (F, J), and pVSV209torYZ (G, K). Panels D to G are images that show red (from the constitutive *rfp*) and green (from the *gfp* reporter) fluorescence and indicate the location of the bacteria in the organ. Panels H to K are images that show only green fluorescence and indicate the level of activity for each reductase operon promoter-*gfp* reporter. Images were taken 72 h postinoculation at 200× magnification. Similar results were observed 24 and 48 h postinoculation. White bars represent approximately 10 μm.

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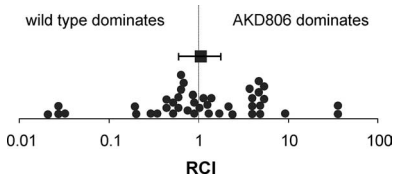

FIG. 3. Competitiveness of AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) during symbiotic colonization. RCI values for squid inoculated with a 1:1 ratio of AKD806 to the wild type, measured 48 h after the exposure of squid hatchlings to the bacterial strains. Each filled circle represents the RCI value calculated from the ratio of strains in an individual animal. The RCI value equals the ratio of AKD806 to ES114 in the host divided by the ratio of these strains in the inoculum, with a value equal to 1 indicating that the strains compete equally well during host colonization. Three independent experiments were performed, and the data were combined for a total of 43 animals. The square represents the average RCI value for the combined experiments, with error bars representing the 95% confidence interval.

The reporter assays described above suggested that certain TMAO reductases are produced during symbiotic colonization. To determine whether the presence of TMAO reductases was important for host colonization, we assayed the ability of AKD806, a *V. fischeri* mutant lacking the three putative TMAO reductases, to colonize aposymbiotic juvenile squid. In experiments where *E. scolopes* squid were exposed to clonal inocula containing either AKD806 or the wild type, the onset of bioluminescence and colonization levels were similar in the two treatments, suggesting that AKD806 does not have a symbiotic defect compared to the wild type (data not shown). However, in some mutants, symbiotic defects are only apparent when they are competed with the wild type in mixed inocula (36, 39). Interestingly, AKD806 displayed no significant colonization defect 48 h after inoculation when competed with the wild type, as evidenced by an average RCI value of 1.05 for three combined experiments (Fig. 3). Similar results were obtained at 72 and 96 h after inoculation (data not shown).

Culture conditions influence TMAO reductase promoter activity. Examination of promoter-*gfp* fusions (above) demonstrated that the promoter strength of the three putative TMAO reductase operons had different expression levels during symbiotic growth and suggested that the expression of each operon might be controlled in response to different signals. To determine whether promoter activity was influenced by growth phase, oxygen tension, medium type, and/or the presence of TMAO, each promoter region was cloned into pAKD701 to generate transcriptional fusions with *lacZ*, and these reporter plasmids were introduced into *V. fischeri* ES114. The resulting strains were then grown under varied conditions (Table 2), with samples harvested for β-galactosidase assays at the mid-log and stationary phases (Table 3). The various conditions were chosen in an attempt to determine how the promoter activity of the putative *V. fischeri* TMAO reductases compared to previously studied organisms under aerobic and anaerobic conditions in the presence and absence of TMAO. In addition, previous work with *Rhodopseudomonas capsulata* determined that TMAO can be used as an electron sink during fermentation rather than as an electron acceptor for anaerobic respiration (22); therefore, media were chosen that were either not fermentable (MS) or contained various fermentable carbon sources (MS with glucose; LBS).

Overall promoter activity was highest for P_{torECA}, with much lower levels for P_{torYZ} and P_{dmsABC}. Upon examining the effect of substrate on promoter expression, we found that TMAO induced P_{torECA} activity during anaerobic growth in both MS and LBS, but under aerobic conditions, TMAO induced P_{torECA} activity only in LBS and to a lesser degree than under anaerobic conditions. Interestingly, the highest levels of P_{torECA} activity were observed during the stationary phase in medium containing TMAO but lacking a fermentable carbon source (MS without glucose) (Table 2). In addition, high levels of P_{torECA} activity were observed during aerobic growth, both with and without TMAO present. In contrast, P_{torYZ} and P_{dmsABC} activities were either unaffected or decreased in the presence of TMAO, with the exception of P_{torYZ} activity during anaerobic growth in LBS.

To address the possibility that the 300-bp promoter fragments for the TorYZ and DmsABC operons did not encompass the essential regulatory region, we constructed reporters containing between 500 and 600 bp upstream of the start codon for each operon. The promoter activity of the larger constructs was compared to that of the 300-bp constructs under a subset of conditions, and no significant differences in promoter activity were observed (data not shown), suggesting that the 300-bp fragments contained the necessary regulatory regions for controlling promoter activity under the conditions tested.

To address the possibility that the measurements of promoter activity could be influenced by the reporter plasmids being multicopy in *V. fischeri* (~10 copies per chromosome) (13), we constructed single-copy chromosomal reporters by cloning the pAKD701-based promoter-*lacZ* fusions into a mini-Tn*7* on pEVS107 (23) and introducing these constructs into *V. fischeri* isolates. This mini-Tn*7* generates site-specific inserts at an intergenic *att* locus downstream of *glmS*, making it a useful tool for creating isogenic chromosomal insertions that do not disrupt other genes (23). The promoter activity of the mini-Tn*7*-based reporters was compared to the plasmid-based reporters under a subset of conditions. For the *torECA* and *dmsABC* reporters, the pattern of activity of the plasmid and chromosomal reporters was similar, although the activity

TABLE 3. Approximate OD_{595} values for cultures harvested for the β-galactosidase assays reported in Table 2

Conditions	OD_{595} values[a]			
	Without TMAO		With TMAO	
	Mid-log phase	Stationary phase	Mid-log phase	Stationary phase
Aerobic				
LBS	1.3	3.7	1.3	3.7
MS with glucose	0.4	1.7	0.4	1.7
MS	0.6	0.9	0.6	0.9
Anaerobic				
LBS	0.3	0.4	0.3	1.3
MS with glucose	0.3	0.9	0.3	2.0
MS	ND	ND	0.4	1.0

[a] ND, not determined; *Vibrio fischeri* will not grow under these conditions.

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was lower overall for the Tn7-based chromosomal reporters (data not shown). In contrast, whereas the plasmid-based *torYZ* reporter assays indicated an increase in activity in response to TMAO when grown in LBS, the chromosomal-based reporter (strain AKD807) showed similar levels of promoter activity in the presence and absence of TMAO (data not shown), suggesting that the increased copy number either affects the activity of an unknown regulatory protein or enhances overall expression such that subtle differences in LacZ activity are easier to detect.

Influence of TorR and FNR on TMAO reductase promoter activity. Previous work by others with *E. coli* and *Shewanella* species has shown that the regulators FNR (11) and TorR (8, 32) influence the expression of TMAO/dimethyl sulfoxide (DMSO) reductase operons. In addition, a recent bioinformatics-based study of *V. fischeri* identified putative FNR binding sites upstream of the *torECA* and *dmsABC* operons, as well as a putative ArcA binding site upstream of *dmsABC* in this organism (28). To determine whether these regulators were involved in the control of promoter activity of the putative TMAO reductase operons in *V. fischeri*, the *lacZ* reporter plasmids were introduced into *V. fischeri* strains JB1, AMJ2 (10), and KV1585, which lack FNR, ArcA, and TorR, respectively. The absence of ArcA did not appear to influence promoter activity in any of the three operons (data not shown). However, FNR and TorR did influence promoter activity of the *torECA* and *dmsABC* operons (Fig. 4). During anaerobic growth, P_{torECA} and P_{dmsABC} activity levels were significantly ($P < 0.05$) lower in JB1 relative to those of wild-type *V. fischeri* (Fig. 4A), suggesting that FNR positively regulates the *torECA* and *dmsABC* promoters, although to low degrees. FNR also positively regulates *dmsABC* in *E. coli* (6); however, FNR does not influence *E. coli torCAD* promoter activity (34) or *Shewanella oneidensis* TMAO reductase gene expression (15).

To determine whether TorR plays a role in the regulation of the putative TMAO reductase promoters in response to TMAO, the promoter activity levels were compared in the KV1585 (the *torR* mutant) background either in the presence or absence of TMAO (Fig. 4B). Interestingly, *torECA* promoter activity increased in the presence of TMAO even without TorR present, although overall promoter activity levels were decreased in the *torR* mutant relative to levels observed in the wild type, suggesting that TorR may be involved in activating *torECA* promoter activity in the absence of TMAO. Additionally, TorR appears to play a role in the negative regulation of *dmsABC* promoter activity, because P_{dmsABC} activity levels were much higher in KV1585 than in the wild type when TMAO was present. Finally, *torYZ* promoter activity does not appear to be significantly affected by FNR or TorR.

Role of TMAO reductases in anaerobic growth. The results of the promoter-reporter assays above suggested that *torECA* encodes the major TMAO reductase in *V. fischeri* under laboratory culture conditions and that either TorYZ and DmsABC do not significantly contribute to TMAO reduction and anaerobic respiration or promoter activity levels are not a good predictor of protein levels and physiological importance. To differentiate between these possibilities, the anaerobic growth of *V. fischeri* strains ES114 (wild type), AKD803 (Δ*torECA* Δ*torYZ*), AKD804 (Δ*torECA* Δ*dmsABC*), AKD805 (Δ*torYZ* Δ*dmsABC*), and AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) was measured in medium that contained TMAO but varied in the presence of a fermentable carbon source (Fig. 5). Under all conditions tested, the presence of TMAO enhanced growth of wild-type *V. fischeri*. In contrast, AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) grown in medium containing TMAO reached densities similar to those of the wild type grown in medium lacking TMAO, suggesting that AKD806 does not produce enzymes capable of reducing TMAO and contributing to growth (data not shown). The growth of the strain containing only TorECA (AKD805) was similar to that of the wild type under all conditions tested, supporting the assumption that TorECA is the major TMAO reductase used during respiratory growth of *V. fischeri* isolates under anaerobic conditions in medium containing TMAO (Fig. 5A to D). In addition, the introduction of the *torECA* locus in *trans* on plasmid pAKD803 to AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*) restored wild-type growth levels during anaerobic growth in LBS and MS containing 40 mM TMAO (data not shown), further supporting the role of TorECA as the major TMAO reductase used by *V. fischeri*

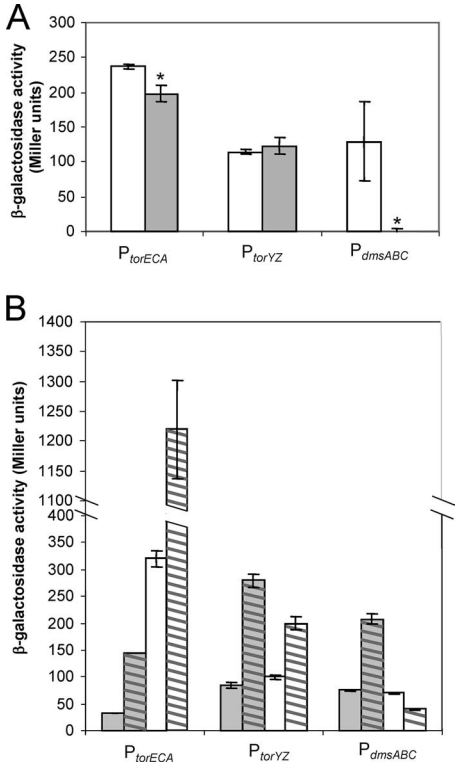

FIG. 4. Influence of FNR and TorR on TMAO reductase promoter activity. (A) β-Galactosidase activity for each putative TMAO reductase promoter-*lacZ* reporter in either wild-type cells (white bars) or JB1 cells (the *fnr* mutant) (gray bars) grown anaerobically in LBS medium. Asterisks indicate statistically significant decreases in activity in JB1 relative to those in the wild type as measured using Student's *t* test (P_{torECA}, $P < 0.02$; P_{dmsABC}, $P < 0.05$). (B) β-Galactosidase activity for each putative TMAO reductase promoter-*lacZ* reporter in KV1585 (the *torR* mutant) grown anaerobically in LBS medium either without (gray bars) or with (gray bars with hatching) 40 mM TMAO and in the wild type grown in LBS medium either without (white bars) or with (white bars with hatching) 40 mM TMAO. The data for the wild type were taken from Table 2. For both panels, error bars represent the standard error of the mean. Experiments were conducted three times, with one representative experiment shown.

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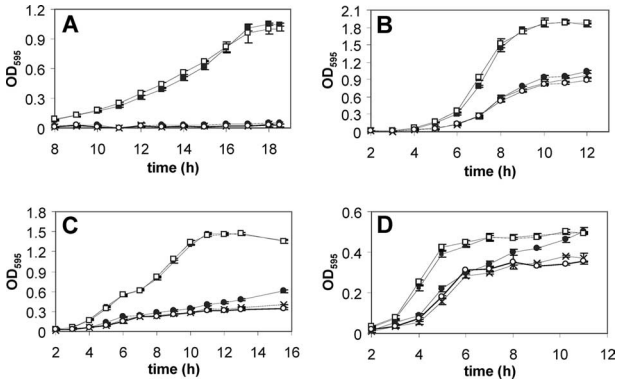

FIG. 5. Anaerobic growth curves of *V. fischeri* TMAO reductase mutants. Wild-type *V. fischeri* and the derived TMAO mutant strains were grown in MS (A), MS with glucose (B), and LBS (rich medium) (C), all containing 40 mM TMAO, or in LBS containing 4 mM TMAO (D). Each point corresponds to the average of three independent replicates. Error bars represent the standard error of the mean. One representative experiment of three is shown. □, *V. fischeri* ES114 (wild type); ×, *V. fischeri* AKD803 (Δ*torECA* Δ*torYZ*); ●, *V. fischeri* AKD804 (Δ*torECA* Δ*dmsABC*); ■, *V. fischeri* AKD805 (Δ*torYZ* Δ*dmsABC*); ○, *V. fischeri* AKD806 (Δ*torECA* Δ*torYZ* Δ*dmsABC*). OD_{595}, optical density at 595 nm.

under these growth conditions. The growth of the strain containing only DmsABC (AKD803) was similar to that of the strain lacking all three operons (AKD806) under all conditions tested, indicating that this reductase is not expressed under these conditions and/or that the main substrate of this enzyme may be a different *N*- or *S*-oxide. The strain containing only TorYZ (AKD804) displays an intermediate growth phenotype, where it appears to contribute to an increase in cell density during entry to the stationary phase in LBS (Fig. 5C) but not in MS (Fig. 5A and B). This is consistent with the reporter data presented in Table 2, where *torYZ* promoter activity is higher during the stationary phase in LBS containing TMAO than in LBS alone, but this effect is not observed in MS medium. These results could reflect a role for TorYZ in the use of TMAO as an electron sink during fermentative growth on compounds found in yeast extract and/or tryptone (LBS) (Fig. 5C and D) but not glucose (MS medium with glucose) (Fig. 5B), as TMAO can play a role in redox balance in *R. capsulata* (22). In addition, TorYZ may be involved in a response to lower concentrations of TMAO because when AKD804 was grown in medium containing 4 mM TMAO, the cell density reached the same level as that of the wild type and AKD805 (Fig. 5D), suggesting that this enzyme's affinity for TMAO may be relatively higher; however, an increase in P_{torYZ} activity was not observed when cells were grown with less than 40 mM TMAO (data not shown).

DISCUSSION

V. fischeri ES114 has a flexible respiratory system and is capable of utilizing numerous compounds as terminal electron acceptors, including oxygen and compounds such as TMAO, nitrate, and fumarate (27). This respiratory flexibility allows *V. fischeri* to thrive in a variety of environments, including free-living lifestyles in the water column or in sediments and in associations with hosts either in mixed gut consortia or in very specific associations with symbiotic partners, such as ES114's colonization of the squid *E. scolopes* (25, 29). In this study, we have laid the groundwork for understanding the role of TMAO reduction in the ability of *V. fischeri* to live both inside and outside of the host.

Because TMAO is commonly found in marine environments, including in the tissue of marine animals, such as squid (5, 21, 41), it would seem likely that utilization of this compound would be important in *V. fischeri* ecology. This assumption is supported by a previous report that identified TMAO reductase activity in the light organ tissues of adult *E. scolopes*, indicating that *V. fischeri* reduces TMAO in the host (27). Although *V. fischeri* can reduce nitrate, and the reduction potential for utilizing the nitrate/nitrite redox pair is greater than that for the TMAO/TMA redox pair (38), published measurements indicate that squid tissue can contain approximately 2,000 times more TMAO than nitrate (20, 21, 41). In addition, the growth rate of *V. fischeri* ES114 in MS containing nitrate is lower compared to growth in medium containing TMAO (27), supporting the idea that although the $\Delta E'_0$ values would suggest that using TMAO as a terminal electron acceptor would be less energetically favorable than using nitrate, TMAO may be an important terminal electron acceptor in *V. fischeri* physiology.

Analysis of the *V. fischeri* ES114 genome sequence revealed the presence of three putative TMAO reductase operons that could contribute to TMAO reduction. These operons show similarity to those found in *E. coli* (Fig. 1); however, although an operon similar to the *E. coli* DMSO reductase-encoding *dmsABC* was identified, *V. fischeri* ES114 cannot respire anaerobically using DMSO as a terminal electron acceptor (27). Moreover, it is difficult to distinguish the *N*- or *S*-oxide substrate for such reductases purely from bioinformatic approaches. Therefore, because the focus of this study was on TMAO reduction, we considered the possibility that *dmsABC* might function as a TMAO reductase in *V. fischeri*. It is worth noting that *V. fischeri* is not able to use TMAO as a sole source of carbon or nitrogen (27), indicating that the use of TMAO by this organism for growth enhancement likely involves respiration.

Based on the fact that fish and squid tissues can contain high levels of TMAO (5, 21, 41), the previous report of TMAO reduction in *E. scolopes* adult light organ tissues (27), the predicted low oxygen tensions in the juvenile and adult *E. scolopes* light organ tissues (30) (our unpublished data), and the observed TMAO reductase promoter activity during colonization (Fig. 2), we predicted that TMAO reductase activity would be an important colonization factor during establishment of the symbiosis; however, based on the results of the colonization assays (Fig. 3), these putative TMAO reductases are apparently not essential for *V. fischeri* to establish colonization of the *E. scolopes* light organ. It is possible that during this time period, oxygen levels in the light organ are sufficient for aerobic respiration and TMAO-based respiration becomes increasingly important as the symbiosis matures and the density of bacteria present in the light organ increases. Alternatively, if oxygen tensions in the light organ are low, as has been predicted (30) (our unpublished data), and aerobic respiration is not being utilized, TMAO may not be present in sufficient

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quantities in the juvenile light organ to support TMAO-based respiration; therefore, this mode of growth is not required for successful colonization. Under this scenario, it is possible that other modes of anaerobic-based energy generation, such as respiration with alternative electron acceptors or fermentation, may be important processes during early colonization. Although we were not able to detect a role for TMAO reduction in our squid colonization assays, this does not rule out the possibility that TMAO reduction plays a role in later stages of the symbiosis and/or in lifestyles outside of the host.

Despite TMAO being common in marine environments (5), the regulation of TMAO reductase activity has not been studied in marine bacteria outside of the genus *Shewanella*. To address this knowledge gap, we studied the regulation of TMAO reductase operons and their relative contribution to TMAO reduction in *V. fischeri*, a member of the *Vibrionaceae* family of marine bacteria. Although *V. fischeri* has a suite of TMAO reductases similar to that of *E. coli*, gene arrangement suggests that regulation of TMAO reductase genes in *V. fischeri* differs from that in other organisms in which it has been studied. Consistent with this hypothesis, the results of this study demonstrate certain differences between regulation of TMAO reductase promoter activity in *E. coli* and that in *V. fischeri*. One striking difference involves the TorCAD and TorECA operon promoter activities. Although in *E. coli* the TMAO reductase system (TorCAD) is expressed under aerobic conditions, the induction of the *torCAD* operon requires the presence of TMAO under either aerobic or anaerobic growth conditions (3). In contrast, the corresponding operon in *V. fischeri* (*torECA*) does not require TMAO for induction, although the addition of TMAO does increase P_{torECA} activity in certain media during anaerobic and aerobic growth (Table 2). In *E. coli*, the increase in *torCAD* promoter activity in response to TMAO is controlled by the TorTSR regulatory system (4, 19, 32) where TorR binds to specific DNA sequences upstream of the *torCAD* operon called *tor* boxes (2). In the absence of TorR, TorCAD is not expressed (26). Similar to those in *E. coli*, TorRS in *S. oneidensis* are involved in positive regulation of TorECAD expression (9), and *tor* boxes are present upstream of *torECAD* (8). Interestingly, canonical *tor* boxes were not identified upstream of *torECA* in *V. fischeri* (data not shown), possibly contributing to the observed differences in promoter activity patterns between these organisms in relation to the presence of TMAO. These differences may be related to the different ecosystems the organisms inhabit, and it is possible that marine host-associated *V. fischeri* encounters TMAO on a more regular basis than *E. coli*, and TMAO reductase activity is required more frequently for optimal growth.

Another notable difference in promoter activity patterns between *V. fischeri* and *E. coli* involves P_{torYZ}. The *torYZ* operon has previously been characterized only in *E. coli*, where it was determined to encode a third TMAO reductase (16). Unlike the *torECA* operon, the *torYZ* operon was determined to be constitutively expressed at low levels under the conditions tested and expression was not induced by TMAO (16). These experiments were conducted using a *lacZ* reporter on a multicopy plasmid (16). In *V. fischeri*, when measured using a multicopy reporter, P_{torYZ} activity appears to be induced in the presence of TMAO when cells are grown anaerobically to the stationary phase in LBS medium (Table 2 and Fig. 4B). However, when P_{torYZ} activity is measured using a chromosomal-based reporter (strain AKD807), TMAO-based induction is not observed (data not shown), suggesting that that the increased copy number either affects the activity of an unknown regulatory protein or enhances overall expression such that subtle differences in LacZ activity are easier to detect. In addition, data from the anaerobic growth curves suggest that TorYZ can contribute to enhanced growth levels in the stationary phase in the presence of TMAO in LBS medium but not in MS medium or in MS medium containing glucose (Fig. 5). Previous work with *R. capsulata* indicated a role for TMAO as an electron sink during fermentative growth (22). LBS medium contains tryptone and yeast extract as carbon and nitrogen sources, and *V. fischeri* is able to grow fermentatively in this medium, as well as in MS containing glucose. It is possible that in *V. fischeri*, TorYZ could be involved in maintaining a redox balance while fermenting compounds other than glucose, contributing to enhanced growth as observed in Fig. 5C; however, this hypothesis will require further testing.

Interestingly, although TMAO reductase activity is not required for early host colonization, the putative TMAO reductase promoters were active during colonization in relative activity levels corresponding to $P_{torECA} > P_{torYZ} \geq P_{dmsABC}$ (Fig. 2). At the time points when the juvenile animals were visualized using epifluorescence microscopy, the bacterial cells are densely packed, which could correspond to the stationary phase in laboratory culture. The promoter activity data for stationary growth in laboratory culture (Table 2) indicate that in most cases, the promoter activity levels fall into a similar pattern of $P_{torECA} > P_{torYZ} \geq P_{dmsABC}$. This pattern of activity levels can be observed in both the presence and absence of TMAO (Table 2) and suggests that although TMAO concentration in the juvenile light organ tissue cannot be determined from these data, no symbiosis-specific pattern of promoter activity is detected in our assays. Future studies analyzing TMAO abundance in *E. scolopes* light organ tissue will be necessary to determine whether this compound is readily available to *V. fischeri* in the light organ.

This study represents the first genetics-based investigation of the contribution of TMAO reductase operons to the utilization of TMAO in the *Vibrionaceae*, and the first in-depth study of TMAO-based respiration in marine bacteria other than *Shewanella* spp., laying the foundation for increasing our understanding of how TMAO reduction contributes to the ecology of marine bacteria. We demonstrate that *V. fischeri* has significant differences from well-studied bacteria in the operon structure and regulation of promoter activity of these reductases. Although the presence of each of the three operons varies between different *Vibrio* species (Fig. 1), the regulatory proteins appear similar and the results from this study may be applicable to other medically and economically important marine-associated *Vibrio* species.

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